Challenging the Wnt Signaling Dogma: Deciphering the Role of Armadillo and Pangolin for Wnt/Wg Target Gene Regulation

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“Кто хочет, тот ищет возможности, кто не хочет - ищет причины.“
(“Where there’s a will, there’s a way. Where there’s no will, there’s an excuse.“)

народная мудрост
(Russian saying)

Zusammenfassung

Eine wesentliche Voraussetzung für die Entwicklung von Tieren sind Signalübermittlungen zwischen Zellen. Eine der wichtigsten Signalmolekülgruppen ist die Wnt/Wg Familie. Wnt/Wg Proteine koordinieren verschiedene Signalkaskaden unter denen der evolutionär hoch konservierte beta-catenin-abhängige Wnt/Wg Signalweg eine herausragende Stellung einnimmt. Diese streng regulierte Signalkaskade ist festgeschrieben im sogenannten „Wnt/Wg Signaling Dogma“. Dieses besagt, dass die transkriptionellen Induktion von Wnt/Wg Target-Genen ausschliesslich durch beta-catenin und TCF/LEF reguliert wird. Aber dennoch, einige wichtige Aspekte zur transkriptionellen Aktivierung der Target-Gene sind immer noch nicht vollständig verstanden und unklar. Bedeutende offene Fragen sind zum Beispiel, i) ob beta-catenin und TCF/LEF tatsächlich die einzigen Mediatoren sind, welche die Transkription der Wnt/Wg Gene regulieren, da viele andere alternative Interaktionspartner von ihnen entdeckt wurden, ii) wie der Transkriptionsfaktor TCF/LEF über *cis*-regulatorische Elemente (Enhancers) die Expression der Target-Gene reguliert und iii) wie zuverlässig synthetische, alternative Aktivatoren des Wnt/Wg Signalweges die transkriptionelle Wnt/Wg Antwort modulieren.

Im ersten Teil der Dissertation beschreibe ich, dass in *Drosophila* Zellen die Wnt/Wg Signalkaskade obligatorisch durch Armadillo und Pangolin (in *Drosophila* beta-catenin und TCF) verläuft. Diese mittels CRISPR/Cas9 in Kombination mit RNA-Sequenzierung und STARR-Sequenzierung gewonnenen Ergebnisse bestätigen das Wnt/Wg Dogma und sprechen gegen alternative Verzweigungen des Singalweges.

Im zweiten Teil der vorliegenden Arbeit bestimme ich die Restriktionen des TCF/Pan Motives in Wnt/Wg Enhancern und schlage vor, dass nicht-konventionelle TCF/Pan Bindungsmotive repressiven Enhancern zu Grunde liegen.

Im letzten Abschnitt analysiere ich schließlich, ob der synthetische und alternative Aktivator des Wnt/Wg Signalweges, GSK3-Inhibitor CHIR99021 (CHIR), die komplette Wnt/Wg Antwort wiedergeben kann. Beim Vergleich der jeweils gefundenen Target-Genen von CHIR und Wg-Medium habe ich herausgefunden, dass CHIR den Wg-stimulierten transkriptionellen Output überraschenderweise

nur zu einem kleinen Teil abbilden kann. Dieses Ergebnis zeigt, dass der Einsatz von CHIR als alternativer Aktivator des Wnt/Wg Singalweges kritisch bedacht werden sollte.

Abstract

A key aspect of animal development is cell-to-cell communication via signaling molecules. One of the most important groups of signaling molecules is the Wnt/Wg family. Wnt/Wg proteins control different signal cascades, an ancient and evolutionarily high conserved beta-catenin dependent pathway being the preeminent example. The details of this tightly regulated signaling cascade are manifested in the Wnt/Wg signaling dogma: The transcriptional induction of target genes exclusively by beta-catenin and TCF/LEF. However, several important aspects of target gene regulation have remained unclear and less well understood. For example, significant open questions are i) whether beta-catenin and TCF/LEF are truly the sole mediators of Wnt/Wg target gene regulation, as alternative binding partners have been reported, ii) how TCF/LEF acts on *cis*-regulatory elements to regulate target gene expression and iii) how reliably synthetic, alternative activators of the Wnt/Wg pathway modulate the transcriptional Wnt/Wg response.

In this thesis, I first show by combining somatic cell genetic engineering (via CRISPR/Cas9 editing) with RNA-sequencing and STARR-sequencing, that in *Drosophila* cells the Wnt/Wg signals proceed obligatorily through Armadillo and Pangolin (the fly's beta-catenin and TCF). These results confirm the veracity of the Wnt/Wg signaling dogma and argue against the existence of distal branches in the Wnt/Wg pathway.

In the second part, I define the sequence constraints of the TCF/Pan motifs that determine the activity of Wnt/Wg-responsive enhancers and propose that non-conventional TCF/Pan binding sites underlie Pan-dependent enhancers with a repressive mode of action.

Finally, in the third part, I analyze whether the GSK3 inhibitor CHIR99021 (CHIR), an alternative inducer of the Wnt/Wg pathway, is able to reproduce a complete Wnt/Wg response. By comparing the genome-wide expression profiles triggered by CHIR and Wg-conditioned medium (WCM), I found that surprisingly CHIR is only able to partially mimic the Wg-triggered transcriptional output. Care must therefore be taken in interpreting and extrapolating from results obtained when using the small inhibitor agent for pathway activation.

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Contents

Chapter 1

Introduction

Wnt/Wg signaling

The fundamental objective of developmental biology is to understand the processes by which organisms grow and develop. Over the years, intensive research has revealed that developmental processes are governed by genetic programs - programs, which are in turn tightly controlled by signaling molecules mediating cell-to-cell communications, for review see (Nusse, 2005). Several families of signaling molecules have been identified, including the Wnt protein family (Wingless (Wg) in *Drosophila*). Wnt/Wg proteins play crucial roles for developmental processes in all multicellular animals ranging from nematodes to humans by specifying the fate and behavior of cells in embryogenesis, pattern formation and adult tissue homeostasis, for review see (Cadigan and Nusse, 1997).

Wnts were identified almost 40 years ago. The first Wnt protein (Int1, now Wnt1) was originally described as preferred integration site of the oncogenic retrovirus MMTV (mouse mammary tumor virus) in virally induced mouse mammary tumors (Nusse and Varmus, 1982), later it was found to encode a homologue of the *Drosophila* segment-polarity gene *wingless* (Rijsewijk et al., 1987) and to cause axis duplications in *Xenopus* embryos (McMahon and Moon, 1989). These early findings revealed already the discrete characteristics of Wnt/Wg signaling including its evolutionary conservation across the animal kingdom, importance for development and connection to oncogenesis.

Today, it is known that the Wnt/Wg signaling pathway controls virtually every aspect of embryonic development, homeostatic self-renewal in adult tis-

sue und when deregulated leads to growth-related pathologies, such as cancer (Nusse, 2005). Wnts trigger a diverse range of signal cascades. The identity of these is dictated by the Wnt ligand and the receptor cohort present on the cell surface. In this thesis I focus on one of these signal cascades - the beta-catenin dependent, canonical Wnt pathway. For simplicity, when I refer to Wnt signaling, I refer to this pathway.

Canonical Wnt/Wg signaling cascade

A simplified schematic of the current model of Wnt/Wg signal transduction is presented in Figure 1. In the absence of Wnt/Wg signals, the transcription of Wnt/Wg target genes is repressed through the interaction of the transcription factor TCF/LEF (T cell factor/lymphoid enhancer factor) with co-repressors such as Groucho (Cavallo et al., 1998) (Roose et al., 1998) and COOP (Song et al., 2010), and the proposed obligate component for transcriptional activation, beta-catenin, is continuously degraded via the ubiquitin-proteasome machinery in the cytoplasm (Kitagawa et al., 1999). Beta-catenin is marked for proteasomal degradation by a large multi-protein complex, the destruction complex. The principal constituents of the destruction complex are Axin, adenomatous polyposis coli (APC), casein kinase 1 alpha (CK1alpha) and glycogen synthase kinase 3 beta (GSK3beta) (Aberle et al., 1997; Ikeda et al., 1998; Hart et al., 1998). While Axin and APC are thought to act as scaffolding proteins, CK1alpha and GSK3beta phosphorylate the N-terminus of beta-catenin, which is decisive for its degradation (Liu et al., 2002).

In the Wnt/Wg ON state, when Wnt/Wg proteins bind to their cognate receptors, the destruction complex of beta-catenin disassembles and dephosphorylated beta-catenin can stabilize and translocate to the nucleus, where it replaces TCF-bound co-repressors to induce the transcription of Wnt/Wg target genes. The Wnt receptors include the seven-pass transmembrane Frizzled (Fz) (Bhanot et al., 1996), which cooperates with a single-pass transmembrane molecule of the LRP family (low density lipoprotein receptor-related protein) (Wehrli et al., 2000; Pinson et al., 2000; He et al., 2000). The mechanism by which the receptor complex transduces the signal leading to the disassembly of the destruction complex, is however still not fully understood and subject of intense research. Several models for Wnt/Wg-induced inhibition of beta-catenin

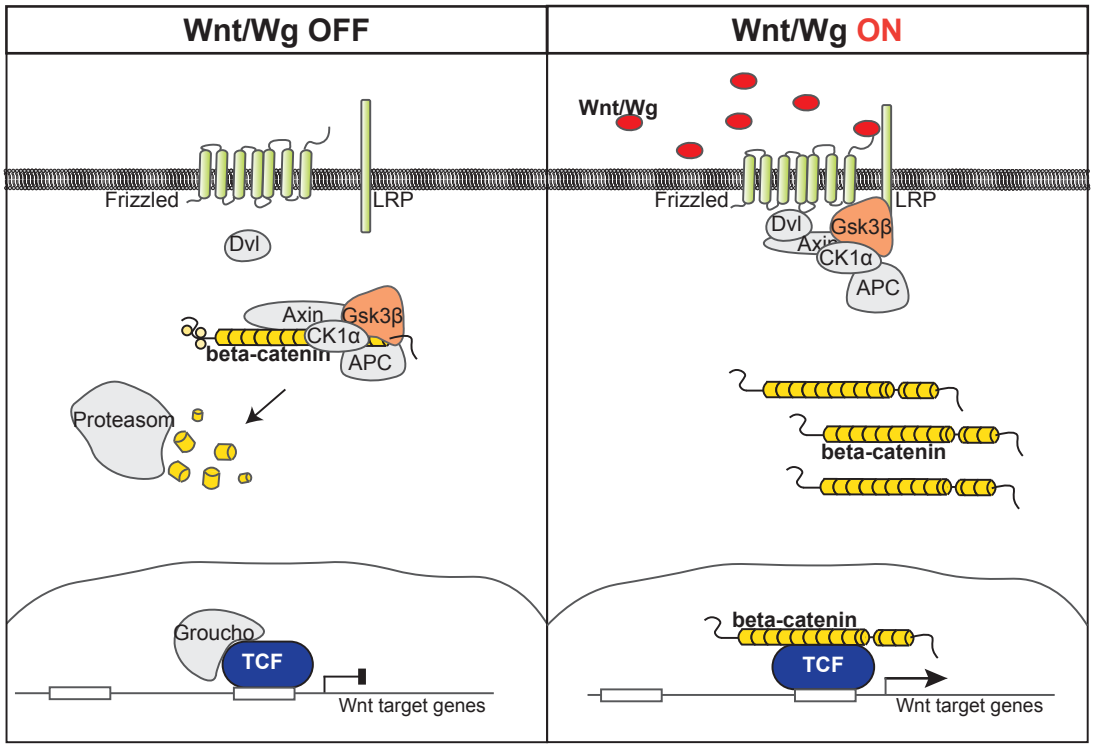

Figure 1: Simplified schematic of the current model of Wnt/Wg signal transduction. In the Wnt/Wg OFF state beta-catenin is constitutively degraded by the destruction complex comprising among others Axin, adenomatous polyposis coli (APC), casein kinase 1 alpha (CK1α), glycogen synthase kinase 3 beta (GSK3β). In the nucleus, Wnt/Wg target genes are repressed by T cell factor/lymphoid enhancer factor (TCF/LEF) family protein interacting with co-repressors such as Groucho. In the Wnt/Wg ON state, the destruction complex is inhibited and beta-catenin translocates into the nucleus, where it displaces TCF-bound co-repressors to induce Wnt/Wg target gene expression. White boxes represent Wnt/Wg responsive elements; LRP, low-density lipoprotein receptor-related protein; Dvl, disheveled.

phosphorylation exist, and are reviewed elsewhere (Macdonald and He 2016).

In the nucleus, the beta-catenin/TCF complex interacts with a variety of transactivator and chromatin-remodeling complexes to activate the transcription of Wnt/Wg target genes. These include the transcriptional co-activators BCL9 (Legless in *Drosophila*) and Pygopus (Pygo) (Stadeli and Basler, 2005; Hoffmans et al., 2005), histone acetyltransferases CBP and p300 (Hecht et al., 2000; Takemaru and Moon, 2000) and chromatin remodeling factor Brg-1 (Barker et al., 2001).

Interaction partners of beta-catenin and TCF

The transcriptional activation of Wnt/Wg target genes by beta-catenin and TCF/LEF transcription factor family is described as Wnt/Wg signaling dogma. However, in addition to TCF/beta-catenin mediated Wnt/Wg signaling, there are speculations in the Wnt/Wg field that alternative transcriptional complexes can also control the Wnt/Wg-triggered gene expression, as various interaction partners for beta-catenin or TCF have been proposed. For example, it was shown that beta-catenin can interact with FOXO transcription factors inducing FOXO-regulated target gene expression (Essers et al., 2005; Hoogeboom et al., 2008; Tenbaum et al., 2012; Liu et al., 2015). Furthermore in mouse embryonic stem cells, it was shown that beta-catenin promotes pluripotency through a TCF-independent mechanism via Oct4 (Kelly et al., 2011). In *Xenopus* it was in addition demonstrated that beta-catenin cooperates with Sox17 inducing the expression of Sox17 target genes (Sinner et al., 2004). More recently it was proposed that beta-catenin forms a complex with YAP1 and TBX5 in human cancer cell lines and this is required for the beta-catenin-driven transformation and tumor maintenance (Rosenbluh et al., 2012). Alternative binding partners have also been reported for TCF, such as Plakoglobin, Mad or Zic (Zhurinsky et al., 2000; Zeng et al., 2008; Murgan et al., 2015). Yet, it remains to be determined, whether these TCF- or beta-catenin-independent interactions can also lead to the activation of Wnt/Wg target genes (see chapter 3.1 on elucidating the requirement of beta-catenin and TCF/LEF for the transcriptional activation of Wnt/Wg target genes in *Drosophila* cells).

TCF/Pan binding sites

Whereas the fly genome encodes a single TCF protein - Pangolin (Pan) (Brunner et al., 1997; van de Wetering et al., 1997) - which operates as repressor in the Wnt OFF state and activator in the Wnt ON state, the vertebrate genome harbors four TCF/LEF genes, with individual TCFs having different functions. For example TCF3/TCF7L1 acts as repressor (Kim et al., 2000; Merrill et al., 2004). All TCF/LEF family members including *Drosophila* Pan recognize the same consensus motif, consisting of CCTTTGATCT in the promoters and enhancers of Wnt/Wg target genes (van de Wetering et al., 1997). This so-called Wnt response element (WRE) is recognized by TCF/Pan via its high mobility group protein domain (HMG) (Chang et al., 2008). Pan and some TCF variants (TCF1/TCF7L and TCF4/TCF7L2) contain an additional domain - the Zinc-

binding C-clamp domain (Ravindranath et al., 2014), that is adjacent to the HMG box and binds to short GC-rich target DNA sequences termed Helper sites increasing the specificity of DNA binding (Atcha et al., 2007; Chang et al., 2008; Hoverter et al., 2014). Several reports have shown that vertebrate TCFs and Pan can also operate in concert with other transcription factors on *cis*-regulatory elements to regulate the transcription of target genes. This suggests a model in which differently regulated enhancers - in a context-dependent manner - contribute to cell-specific Wnt/Wg target gene expression. For example, Pan has been found to co-occupy enhancers with other transcription factors such as Mad, Doc2, Pnr, and Tin during dorsal mesoderm specification in *Drosophila* embryos (Junion et al., 2012), and vertebrate TCF4/TCF7L2 has been shown to co-localize on *cis*-regulatory DNA elements with SMAD1 to regulate hematopoietic differentiation and regeneration (Trompouki et al., 2011). TCF4/TCF7L2 also co-operates with the transcription factor CDX2 in colonic cells (Verzi et al., 2010). However, how co-occupied enhancers contribute to target gene regulation is still not fully understood (see chapter 3.2 about the motif requirements for Wnt/Wg-responsive enhancers).

Wnt/Wg target genes

Since Wnt/Wg target genes have been found to be expressed in a cell-type specific and context-dependent manner it is anticipated that a "universal" set of target genes may not exist, for review see (Clevers, 2006). An updated and ever-growing list of Wnt/Wg target genes can be found at `https://web.stanford.edu/group/nusselab/cgi-bin/wnt/target_genes`. However, the list is still far from complete. Certainly the discovery of targets is challenging since their expression is spatiotemporal and cell-type specific (Ramakrishnan and Cadigan, 2017) (see chapter 3.1 for the identification of *Drosophila* Kc167 Wnt/Wg target genes).

Among the list of Wnt/Wg target genes, two target genes are especially prominent, *c-myc* (He et al., 1998) and *cyclinD1* (McCormick and Tetsu, 1999), as they contribute to malignant growth when deregulated. Other important Wnt/Wg target genes comprise a list of Wnt/Wg pathway components, such as *axin2* (Jho et al., 2002), *fz* (Cadigan et al., 1998; Sato et al., 1999), *arrow* (Wehrli et al., 2000) and *TCF* and *LEF1* (Roose et al., 1999; Hovanes et al., 2001), providing an important feedback mechanism for tuning the pathway

(Perrimon and McMahon, 1999). Other important regulatory targets are *naked cuticle* (Zeng et al., 2000; Rousset et al., 2001), *notum* (Gerlitz and Basler, 2002) and *nemo* (Zeng and Verheyen, 2004), which act as Wnt/Wg pathway antagonists. In our study, we identified *Toll-7* as interesting candidate Wnt/Wg target gene, which we anticipate also to play a role in Wnt/Wg signaling (see chapter 3.1.2 for the functional characterization of Toll-7).

Wnt/Wg-mediated repression

In contrast to the well-studied mechanism of gene activation, the mechanisms by which beta-catenin and TCF/LEF promote target gene repression are poorly understood (Affolter et al., 2008). While reports have shown that TCF is not required for the repression of the Wnt target gene *HesX1*, as beta-catenin mediates repression together with Pros1 (Olson et al., 2006), other studies proposed that TCF/Pan, when complexed with Mad mediates Wnt/Wg target gene repression in *Drosophila* without Armadillo (Arm) (Zeng et al., 2008). The study even suggested that Mad competes with Arm for TCF/Pan binding (Zeng et al., 2008). Furthermore it is not clear, in which context traditional or alternative TCF/Pan binding sites are used: An alternative TCF/Pan binding site AGAWAW (W=A/T) has been reported to mediate the repression of the Wnt/Wg target gene *Ugt36Bc* (Blauwkamp et al., 2008), but it has also been shown that for several target genes TCF/Pan can repress via traditional TCF/Pan binding sites. Examples include *E-cadherin* in mouse keratinocytes, *stripe* in the *Drosophila* epidermis or *dpp* in the fly leg imaginal disc (Piepenburg et al., 2000; Jamora et al., 2003; Theisen et al., 2007). A recent study proposed that a Helper-like motif (KCCSSNWW [K=G/T, S=G/C, N=any base, W=A/T]), which has been found to co-occur with the alternative TCF/Pan binding site, may mediate the repression of *Tiggrin* in *Drosophila* (Zhang et al., 2014). This suggests that TCF/Pan may also act via a distinct bipartite mechanism to regulate Wnt/Wg-repressed target genes. Thus, several models have emerged for a Wnt/Wg-mediated repressive mode of action but more studies are needed to gain a comprehensive understanding of the mechanism (see chapter 3.2 on analyzing the motif content of repressive Wnt/Wg-responsive enhancers).

Modulators of the Wnt/Wg pathway

Given the importance of Wnt/Wg signaling in developmental processes and adult tissue homeostasis throughout life, it is not surprising that aberrant

Wnt/Wg signaling has been implicated in several growth-related pathologies, including degenerative diseases as well as cancer, for review see (Nusse, 2005). Many synthetic agents targeting the Wnt/Wg pathway as inhibitory drugs or as agonists have been developed. While various pathway-inhibiting drugs impede the function of Axin, the vast majority of pathway-activating agents target GSK3, for review see (Nusse and Clevers, 2017). However, a caveat when modulating the Wnt/Wg pathway with synthetic agents is that other cellular processes may be affected. For example, GSK3 is involved in a diverse spectrum of molecular processes including insulin signaling, microtubule regulation, inflammatory pathways, and developmental programs such as Hedgehog and Notch signaling (Doble, 2003). Yet, it is unknown how reliably GSK3 inhibition mimics Wnt/Wg pathway activation (see chapter 3.3 on investigating the transcriptomic profiles triggered by the synthetic GSK3 inhibitor CHIR99021 in comparison to the expressional profile triggered by Wingless-ligand).

Chapter 2

Aim of the Thesis

The aim of the thesis is to comprehensively determine how Wnt/Wg target genes are regulated. Using *Drosophila melanogaster* as a model system and a combination of large-scale quantitative high-throughput sequencing, computational, genetic and biochemical approaches, I set out to address the specific aims listed below to add some pieces to the still incomplete picture of nuclear Wnt/Wg signaling. The findings are summarized in the form of manuscripts in the chapter "Results and Discussion".

1. Probing the canonicity of the Wnt/Wg signaling pathway, to investigate whether the Wnt/Wg signaling pathway can control Wnt/Wg target genes in the absence of Armadillo or Pangolin (chapter 3.1).
    a. Establishing a protocol for the generation of clonal Cas9-edited *Drosophila* cells to be used in Aim 1 (chapter 3.1.1).
    b. Characterization of in Aim 1 identified Wnt/Wg target gene *Toll-7* to determine its role for Wnt/Wg signaling (chapter 3.1.2).
2. Deciphering the code of the Wnt/Wg-responsive enhancers identified in Aim 1 to gain insights into the TCF/Pan motif requirements for the activity of enhancers (chapter 3.2).
3. Analyzing the transcriptional output triggered by GSK3beta inhibition by using the alternative Wnt/Wg pathway activator CHIR, to elucidate whether it reliably restates the Wnt/Wg response (chapter 3.3).

Chapter 3

Results and Discussions

3.1 Probing the canonicity of the Wnt/Wingless signaling pathway

In this section, we addressed the question of whether either of the canonical transduction components, beta-catenin or TCF, can be bypassed when the Wnt/Wg pathway is activated.

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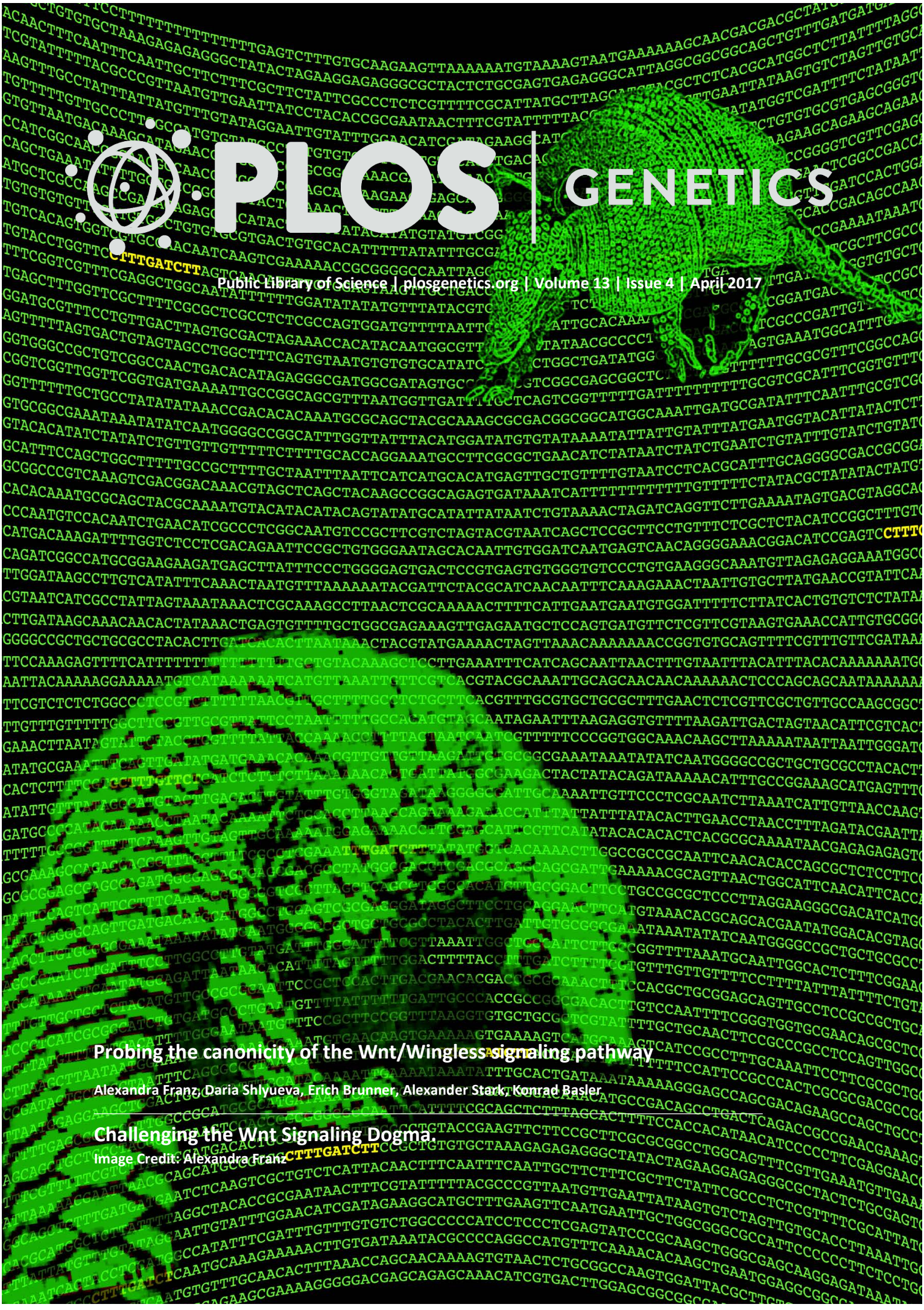
Our blog post about "Understanding Images: Challenging the Wnt signaling dogma" on PLOS Biologue can be found here: `http://blogs.plos.org/biologue/2017/07/03/understanding-images-challenging-the-wnt-signaling-dogma/`



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Challenging the Wnt Signaling Dogma.

Image Credit: Alexandra Franz

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RESEARCH ARTICLE

Probing the canonicity of the Wnt/Wingless signaling pathway

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Abstract

The hallmark of canonical Wnt signaling is the transcriptional induction of Wnt target genes by the beta-catenin/TCF complex. Several studies have proposed alternative interaction partners for beta-catenin or TCF, but the relevance of potential bifurcations in the distal Wnt pathway remains unclear. Here we study on a genome-wide scale the requirement for Armadillo (Arm, *Drosophila* beta-catenin) and Pangolin (Pan, *Drosophila* TCF) in the Wnt/Wingless(Wg)-induced transcriptional response of *Drosophila* Kc cells. Using somatic genetics, we demonstrate that both Arm and Pan are absolutely required for mediating activation and repression of target genes. Furthermore, by means of STARR-sequencing we identified Wnt/Wg-responsive enhancer elements and found that all responsive enhancers depend on Pan. Together, our results confirm the dogma of canonical Wnt/Wg signaling and argue against the existence of distal pathway branches in this system.

Author summary

Our manuscript addresses the question of whether either of the canonical transduction components, beta-catenin or TCF, can be bypassed when the Wnt pathway is activated. By using somatic cell genetics in *Drosophila* cells (via CRISPR/Cas9 editing) in combination with RNA-seq and STARR-seq (Self-transcribing-active-regulatory-region-sequencing) as functional read-outs, we provide firm evidence against the existence of distal branches in the Wnt pathway.

Introduction

Wnt proteins are highly conserved signaling molecules specifying the fate and behavior of cells in multicellular animals ranging from nematodes to humans [1]. They play crucial roles in embryogenesis, pattern formation and tissue homeostasis during development and in adult life. Therefore it is not surprising that aberrant Wnt signaling has been found to be implicated in many human diseases [2].

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Following the identification of Wnt proteins nearly 40 years ago [3–5] genetic and biochemical studies have revealed mechanistic details of how the signaling cascade operates when cells receive a Wnt signal [for review see 6]. As a consequence of Wnt/Wg proteins binding their cognate receptors, beta-catenin is no longer marked for degradation and accumulates in the cytoplasm and nucleus [7–10]. In the prevailing model, TCF is targeted through its DNA binding domain to Wnt-responsive elements (WREs) in the promoters or enhancers of target genes [11] and initiates the transcription of Wnt/Wg-responsive genes when complexed with beta-catenin. In the absence of Wnt/Wg ligand, beta-catenin is phosphorylated and degraded while TCF is bound by transcriptional repressors, such as Groucho and Coop [12–15]. In contrast to the well-studied mechanism of gene activation, the mechanisms by which beta-catenin and TCF promote target gene repression are not well understood [16]. Several reports suggest that, in addition to beta-catenin and TCFs, other factors are involved in Wnt-mediated repression, such as Prop1, Mad or Zic [17–19]. Furthermore it is not clear, in which context alternative [20] or traditional TCF binding sites are used for transcriptional repression [21–23].

A recent study showed that TCF4 is a predominant factor in mediating the Wnt response and for recruiting beta-catenin to DNA [24], however ongoing research on the Wnt signaling pathway has repeatedly demonstrated that beta-catenin as well as TCF interacts with various other proteins. Yet it remains to be determined, whether alternative transcriptional complexes also regulate the expression of Wnt/Wg target genes. For example, an interaction between beta-catenin and FOXO-transcription factors in mouse and DLD-1 human colon carcinoma cells has been demonstrated resulting in the activation of genes involved in oxidative stress and colon cancer metastasis [25–27]. Furthermore in mouse embryonic stem cells it was shown that beta-catenin forms a complex with Oct4 to promote Oct4-driven transcription and pluripotency [28]. In addition, studies in *Xenopus* reported an interaction between beta-catenin and Sox17, promoting expression of Sox17 target genes [29], and more recently it was suggested that beta-catenin complexes with YAP1 and TBX5 in human cancer cell lines [30]. In addition, alternative binding partners have also been reported for TCF, such as Plakoglobin or Mad [31, 18].

In this study, we address the question of whether alternative routes exist that bypass beta-catenin or TCF to promote the transcription of Wnt/Wg target genes in *Drosophila* cells. Using cells that lack either Arm or Pan and functional read-outs (i.e. RNA-seq and STARR-seq), we show that both, Arm and Pan, are absolutely required for target gene activation and repression. Consistent with these findings, we further demonstrate that Wnt/Wg-responsive enhancers also require Pan, arguing against the existence of distal branches in the Wnt signaling pathway.

Results

Genome-wide identification of Wnt/Wg target genes by RNA-sequencing

Next-generation RNA-sequencing (RNA-seq) was used to identify and quantify the expression of target genes of the Wnt/Wg signaling pathway in *Drosophila* Kc167 cells. Cells were treated either with Wg-enriched medium (referred to as Wingless-conditioned medium, WCM; [32]), or control-conditioned medium (CM) lacking the Wg ligand. Wg-responsive genes were determined by statistical analysis of gene expression levels in treated samples versus control samples, according to a protocol described in [33]. In order to determine a high confidence set of Wnt/Wg targets, genes had to pass the following selection criteria: exhibit a significantly altered expression profile (WCM vs CM, p-value ≤ 0.0005) and an at least two-fold change of expression upon Wg stimulation (Fig 1A). WCM-treatment resulted in the robust induction of 51 genes. Among them we found previously identified Wnt/Wg target genes such as *naked*

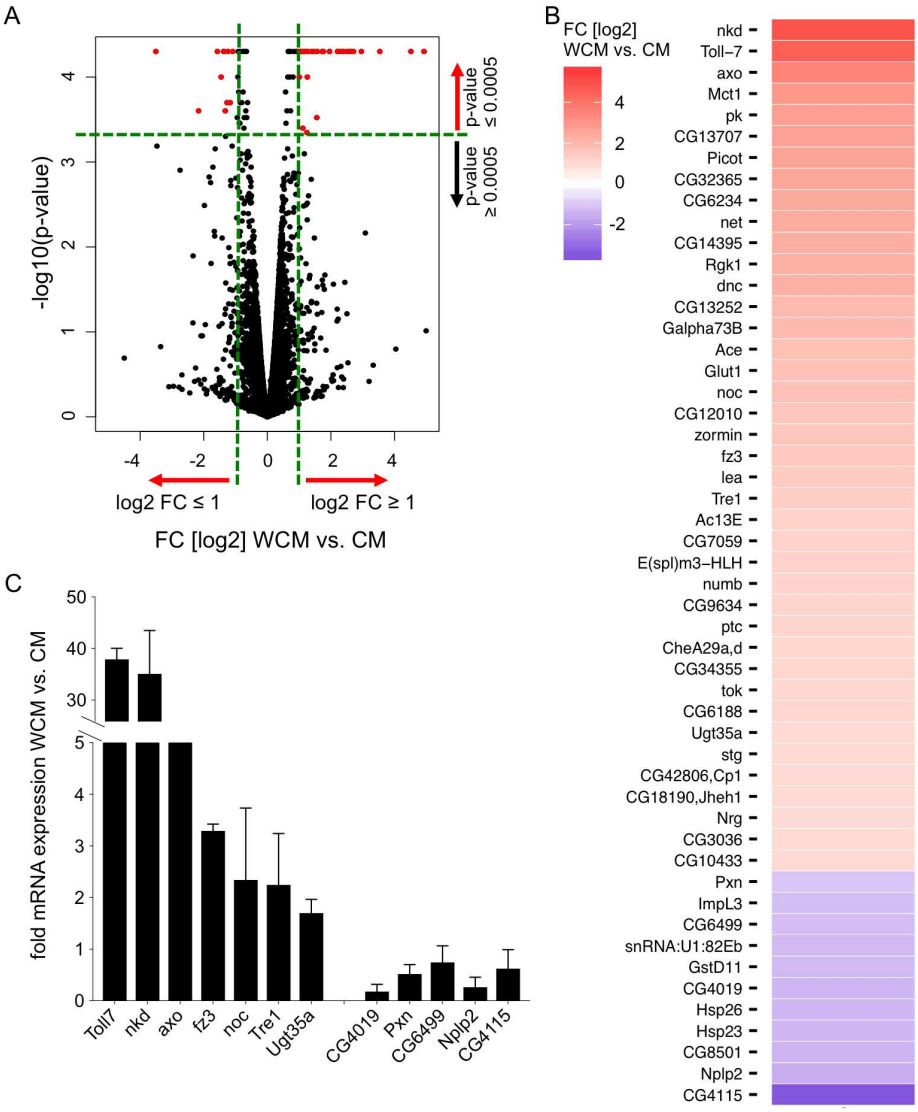

Fig 1. Identification of Wnt/Wg-responsive genes. (A) Volcano plot showing the log2 fold change (x-axis) and statistical significance (y-axis; -log10 p-value) of Wnt/Wg-responsive genes. 51 genes were significantly differently expressed after WCM-treatment (red dots), 40 genes showed an up-regulation (positive targets), 11 genes were down-regulated (negative targets) after WCM treatment. Black dots represent all genes that showed an altered expression profile. (B) Heatmap showing the log2 fold change (FC) of expression of the 51 Wnt/Wg-responsive genes in wild-type cells (WT). The expression profile of positive target genes is depicted in red, the expression profile of negative targets is in blue. (C) Confirming of candidate genes using qRT-PCR. *Drosophila* Kc cells were stimulated with WCM or CM for 24 h. Analysis of expression profiles of candidate target genes (7 positive and 5 negative) after treatment versus control confirmed their induction after WCM stimulation.

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cuticle (*nkd*), *CG6234*, *frizzled 3* (*fz3*) and *Peroxidasin* (*Pxn*) [34–36, 20], confirming our quality filters. 40 genes were at least two fold up-regulated (positive targets) and 11 genes two fold down-regulated (negative targets) (Fig 1B). 7 positive and 5 negative candidate target genes were confirmed by qRT-PCR (Fig 1C). This high confidence set of Wnt/Wg target genes was

used to systematically elucidate potential beta-catenin or TCF-independent branches of Wnt/Wg signaling.

An absolute requirement for Armadillo for activation and repression of Wg target genes

To investigate whether Arm can be bypassed via alternative branches of the pathway, we generated *arm* knockout cells using the CRISPR/Cas9 technology as described by Bassett and colleagues [37]. In order to generate *Drosophila arm* null mutant cells we used sgRNAs targeting two different exons that are present in all transcript variants (Fig 2A and 2B). sgRNA-a1 on the reverse strand targets the translational start site residing in exon 2. sgRNA-a2 targets a site in the third exon. The presence of CRISPR-induced mutations generated by NHEJ (non-homologous end joining) was assessed by sequencing of the PCR products spanning the sgRNA target sites (see Material and Methods). The analysis revealed that most of the alleles had indel mutations at the expected cleavage sites, some of which lead to the deletion of the translational start site or to frameshifts in exon 3. To generate an $arm^{-/-}$ cell line, we carried out serial dilutions and searched for cell populations that carried previously identified mutations using allele-specific primers as described in [38]. In this way, we isolated an *arm* null mutant cell line (named $arm^{-/-AFII7/8}$) which was a homogenous cell population (see Material and Methods) carrying a deletion of either one or sixteen nucleotides in the second exon, each of them destroys the START codon (ATG), and a deletion of one nucleotide in the third exon (Fig 2B). Importantly no wild-type alleles were present. These mutations, affecting both *arm* alleles, result in frameshift mutations introducing a premature termination codon that should trigger nonsense-mediated mRNA decay (NMD) [39] (S1A Fig). We confirmed the complete loss of Arm protein in $arm^{-/-AFII7/8}$ cells by Western blot analysis (Fig 2C, S1B Fig).

Next we investigated whether Arm is absolutely required for the Wnt/Wg-driven transcriptional output. To that end $arm^{-/-AFII7/8}$ cells were treated either with WCM or CM and target gene responses were monitored by RNA-seq. We found that the induction of the positive Wnt/Wg target genes is dependent on Arm, since their expression was not changed in *arm* null mutant cells. Similarly all negative target genes are no longer repressed in $arm^{-/-AFII7/8}$ cells (Fig 3A and 3B). These results demonstrate that Arm is absolutely necessary for both, activation and repression of identified Wnt/Wg targets. We confirmed our results with qRT-PCR analysis of 11 candidate targets genes (S2 Fig).

Requirement of Pangolin for transcriptional regulation of Wg target genes

From the analysis above, we conclude that Arm is absolutely required for both activation and repression of Wnt/Wg target genes and interpret this as evidence against the existence of an Arm-independent Wnt/Wg signaling transcriptional output. Since several alternative interaction partners for beta-catenin have been proposed for the activation and the repression of genes, such as Sox17 [29], Oct4 [28] and Prop1 [17], we next asked whether TCF-independent Wnt/Wg signaling exists. To search for TCF-independent Wnt/Wg signaling, we utilized a similar setup as described above to generate *pan* null mutant cells. Two distinct sgRNAs were used to target independent loci within the *pan* gene (Fig 4A). We isolated a population of *pan* null mutant cells that no longer contain any wild-type allele. Similar to the $arm^{-/-AFII7/8}$ cells, the selected *pan* null mutant cells, termed $pan^{-/-AF1AD26}$, carry three defined mutations that lead to frameshift mutations. Molecular analysis of the alleles revealed no wild-type allele but a large deletion of approximately 9 kb spanning the two selected CRISPR sites (Fig 4B). In addition, $pan^{-/-AF1AD26}$ cells also harbor two distinct frameshift mutations in the HMG box, both

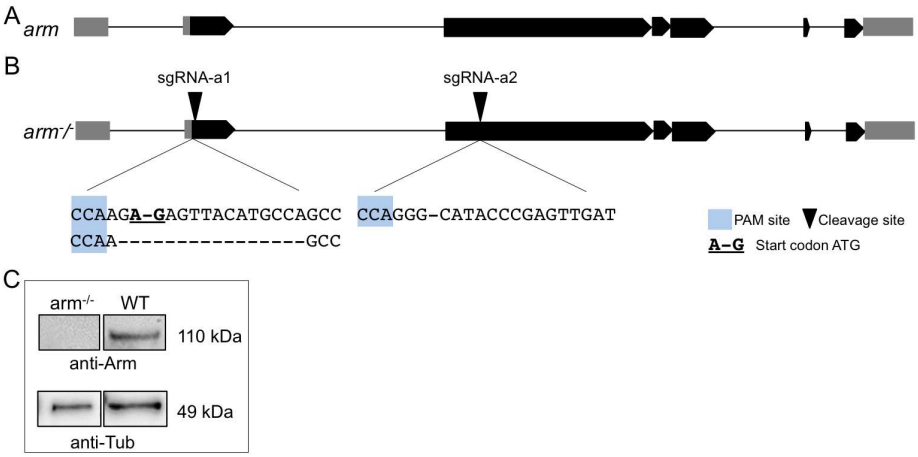

Fig 2. Mutagenesis of the *arm* gene. (A) Schematic of the *arm* gene-locus. Untranslated regions (UTR) are indicated in grey boxes, translated exons in black. (B) CRISPR targeting strategy: Target sites of both sgRNAs are represented by black triangles. The PAM site is highlighted in blue. Sequences as they are present in arm$^{-/-AFII7/8}$ (arm$^{-/-}$) cells are depicted below. Bold and underlined nucleotides represent the TSS. (C) Western blot analysis using an α-Arm antibody on lysates from wild-type (WT) and arm$^{-/-AFII7/8}$ (arm$^{-/-}$) cells. As expected the arm$^{-/-AFII7/8}$ (arm$^{-/-}$) cells are devoid of Arm protein. Tubulin was used as loading control.

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of which result in premature termination codons (S3A Fig) and NMD. Consistent with this qRT-PCR analysis showed a reduction of *pan* mRNA in knockout cells compared with wild-type cells (S3B Fig). The presence of the three *pan* mutant alleles suggests that at the *pan* locus Kc cells are polyploid; segmental polyploidy has been reported for Kc cells [40]. Since no anti-Pan antibodies were available to confirm the absence of functional Pan protein we used the *wingful* luciferase reporter assay, an artificial built reporter giving a robust and high Wg-response [41]. Consistent with the absence of Pan, in pan$^{-/-AF1AD26}$ cells the *wingful* reporter could no longer be induced after WCM-stimulation; responsiveness could be restored by Pan overexpression (Fig 4C and 4D).

To answer the question of whether Pan is dispensable for Wnt/Wg-regulated induction of target genes, we treated pan$^{-/-AF1AD26}$ cells with either WCM or CM and performed RNA-seq. We observed that pan$^{-/-AF1AD26}$ cells can no longer transduce the Wnt/Wg signal as expression of none of the identified Wnt/Wg targets was altered. Neither positive nor negative Wnt/Wg-target genes significantly changed their expression profile in *pan* knockout cells after Wg stimulation providing evidence that Pan is indispensable for the activation and repression of Wnt/Wg target genes (Fig 5A and 5B). The lack of a change in the expression of several candidate Wnt/Wg targets was confirmed by qRT-PCR (S2 Fig).

De-repression in the absence of Pan

Like most major developmental signaling pathways, the Wnt/Wg system uses a "transcriptional switch" mechanism to positively regulate target gene expression [42]. In the absence of Wnt/Wg signaling, the transcription of target genes is repressed by Pan via its interaction with co-repressors such as Groucho or Coop [13, 15]. Pan turns into an activator when complexed with Arm following pathway activation. It has been shown that loss of Pan function leads to de-repression of the Wg target genes *nkd* and *CG6234* in the Wg OFF state *in vivo* and *in vitro* [35, 43]. To determine whether this mode of action is valid for the entire set of identified Wnt/Wg target genes we compared the gene expression profiles of wild-type and pan$^{-/-AF1AD26}$ cells

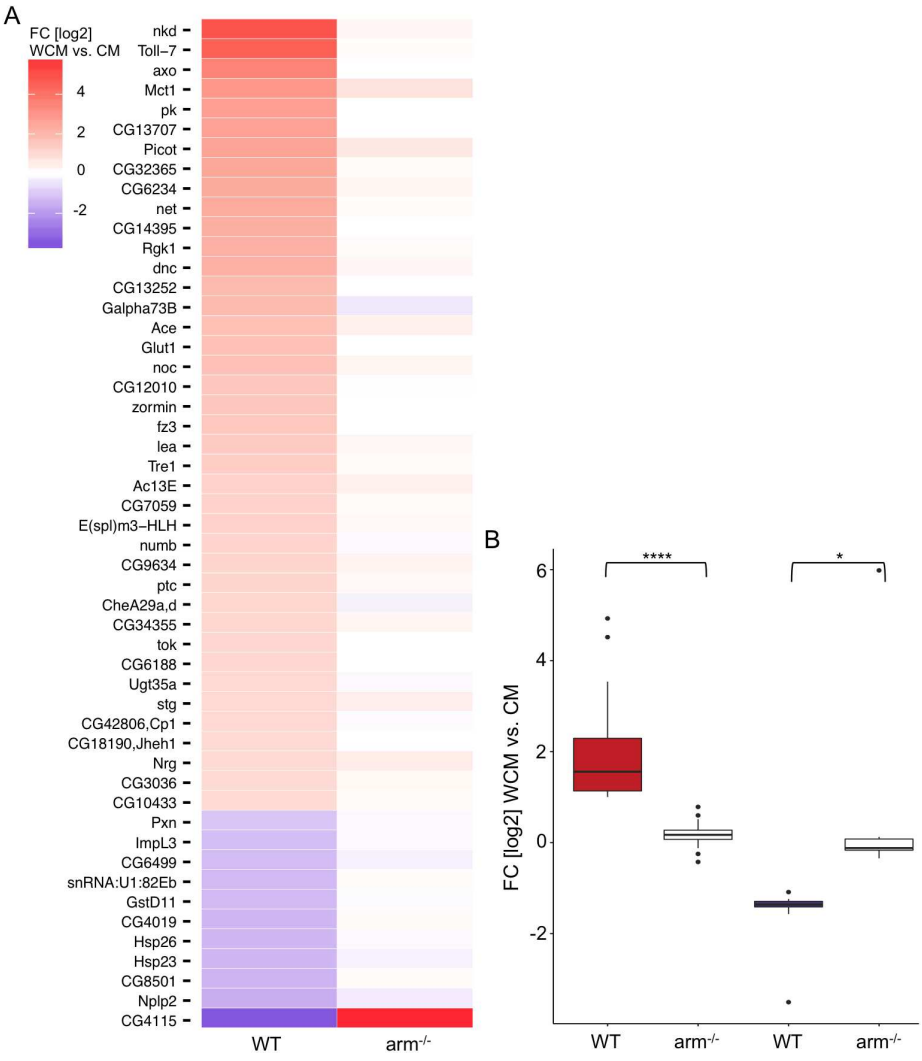

Fig 3. Gene expression analysis of Wg/Wnt target genes in arm$^{-/-AFII7/8}$ cells. (A) Heat map of Wnt/Wg target genes showing their log2 fold change (FC) of expression after to before WCM treatment in wild-type (WT) and arm$^{-/-AFII7/8}$ (arm$^{-/-}$) cells. The genes are listed according to the strength of the induction of their expression in WT cells. Strongest up-regulated genes are on top. Up-regulated genes are shown in red, down-regulated genes in blue, no expression in white. (B) Boxplots showing the difference in gene activity for up- and down-regulated genes after WCM stimulation in wild-type (WT) and arm$^{-/-AFII7/8}$ (arm$^{-/-}$) cells. Paired t-test: * $\leq$ 0.05, *** $\leq$ 0.0001.

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in the absence of Wnt/Wg signaling. Interestingly, we found that only a fraction (37.5%) of positive target genes were de-repressed in *pan* null mutant cells (Fig 5C; fold change $\geq$ 2; p-value $\leq$ 0.0005); among them were *nkd* and *CG6234* [35]. We also noted that this set of de-repressed genes is highly induced in the presence of Wg ligand (Fig 5C). In contrast, the absence of Pan had no effect on the basal expression of the other (the majority) target genes. However, we also identified some genes exhibiting reduced levels of expression in unstimulated *pan* knockout cells (Fig 5C), suggesting that Pan might be required for their transcription in the absence of Wnt/Wg signaling. Blauwkamp and colleagues (2008) proposed this mode of

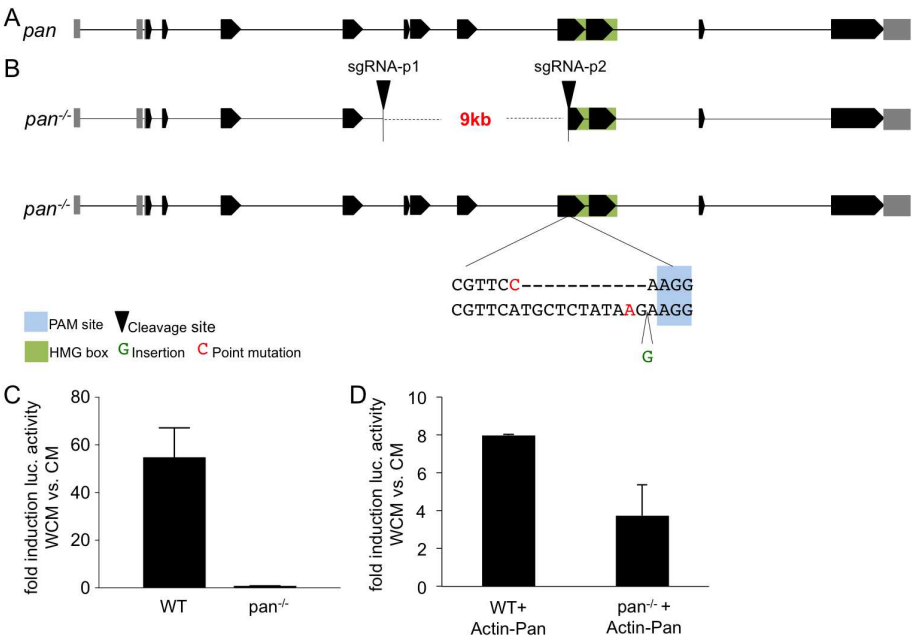

Fig 4. Mutagenesis of the *pan* gene. (A) Schematic of the *pan* gene-locus. Untranslated regions (UTR) are indicated in grey boxes, translated exons in black. HMG box in green. (B) CRISPR targeting strategy: Target sites of both sgRNAs are represented by black triangles. The PAM site is highlighted in blue, the HMG box in green. Sequences as they are present in the pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells are depicted below. (C) Wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells were transfected with the *wingful* luciferase reporter expression vector and Renilla expression vector 24 h prior stimulation with WCM (as control CM was used). After 24h stimulation, reporter activity was analyzed. (D) Wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells were transfected with Pangolin overexpression vector under the control of the Actin promoter together with *wingful* luciferase reporter expression vector and Renilla expression vector 24 h prior stimulation with WCM (as control CM was used). After 24h stimulation, reporter activity was analyzed.

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action for Pan in *Drosophila* Kc cells for several negative target genes, when cells were not exposed to Wnt/Wg [20].

Genome-wide identification of Wnt/Wg-responsive enhancers

Transcription factors bind to specific signal responsive elements in the promoters or enhancers of target genes in order to regulate their expression [44]. So far we have analyzed in detail the Wnt/Wg-triggered transcriptional output and demonstrated that both, Arm and Pan are absolutely required for the activation and repression of Wnt/Wg target genes in *Drosophila* cells. However, in order to obtain a more complete understanding of the transcriptional regulation of Wnt/Wg target genes, we carried out Self-transcribing-active-regulatory-region-sequencing (STARR-seq), a genome-wide enhancer activity assay that reveals the identity of DNA sequences that can function as enhancers in a particular cell type [45–46] and in response to external stimuli, such as the insect steroid hormone ecdysone [47]. To identify enhancers whose activity changes in response to the Wnt/Wg signal, we performed STARR-seq under conditions of active Wnt/Wg signaling and under control conditions (Fig 6A, S4A Fig). For technical reasons, we used the Gsk3β-inhibitor CHIR99021 (CHIR)–a widely used alternative inducer of Wnt-signaling to stimulate Wg signaling in the STARR-seq experiments [48, 49] (see Material and Methods), whose activity we compared to WCM by using the

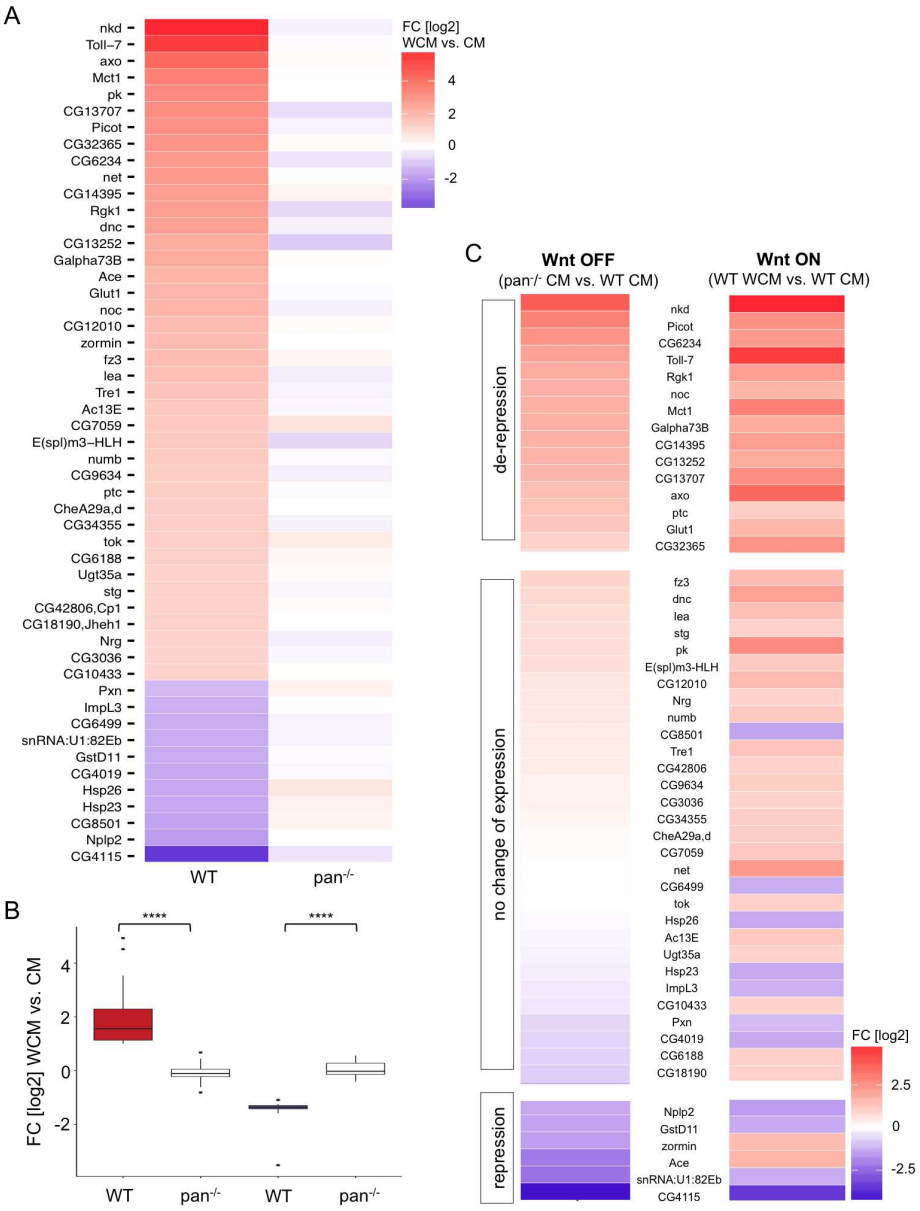

Fig 5. Gene expression analysis of Wnt/Wg target genes in pan$^{-/-AF1AD26}$ cells. (A) Heat map of Wnt/Wg target genes showing their log2 fold change (FC) of expression after WCM treatment versus control treatment in wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells, respectively. The genes are listed according to their intensity of induction in WT cells. Strongest up-regulated genes are on top. Up-regulated genes are shown in red, down-regulated genes in blue, no expression in white. (B) Boxplots showing the difference in gene activity for up- and down-regulated genes after WCM stimulation in wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells. Paired t-test: *** $\leq$ 0.0001. (C) Gene expression analysis of Wnt/Wg target genes in the Wnt OFF and Wnt ON state. Heat map of log2 fold change (FC) according to the genotype (pan$^{-/-AF1AD26}$ (pan$^{-/-}$) CM vs. wild-type (WT) CM) or WCM treatment (wild-type WCM vs. wild-type CM). The genes are listed according to their expression levels in the Wnt OFF state. Up-regulated genes are in red, down-regulated genes in blue, no expression in white. De-repression: fold change (log2) $\geq$ 1, p-value $\leq$ 0.0005, repression: fold change (log2) $\leq$ -1, p-value $\leq$ 0.0005.

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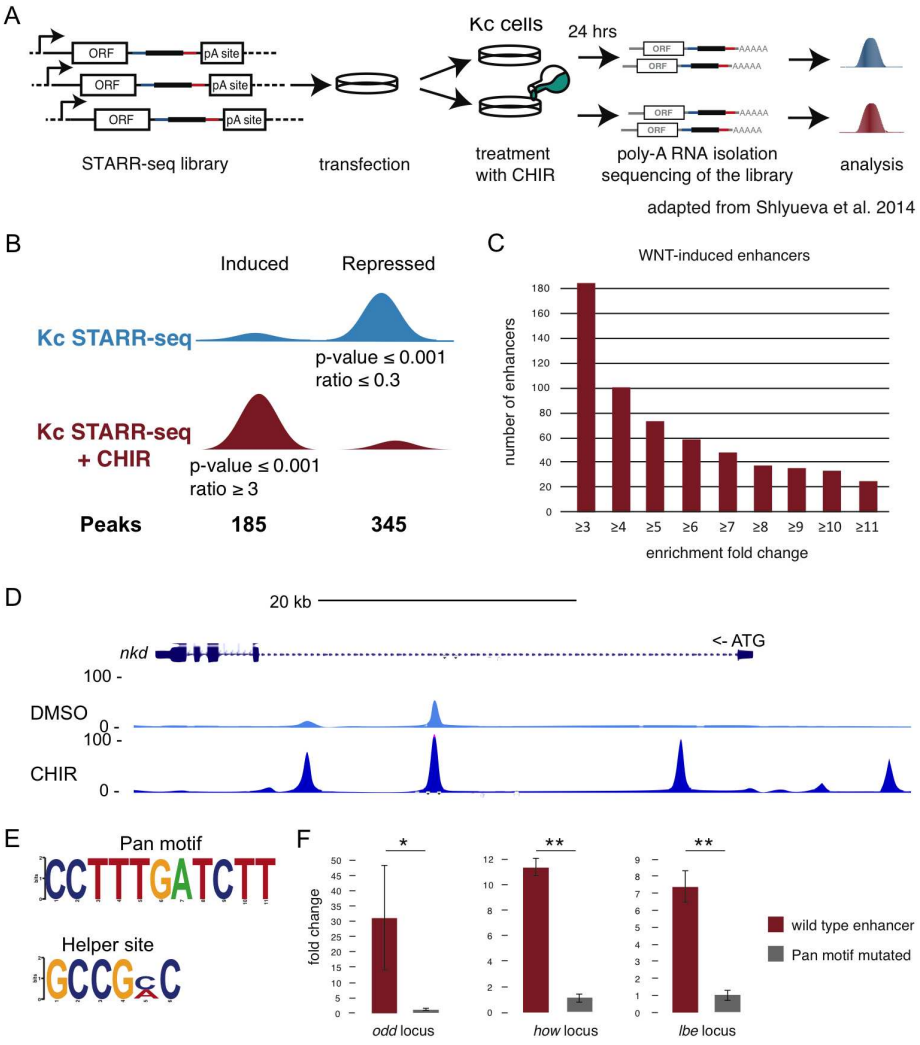

Fig 6. Identification of Wnt/Wg-responsive enhancers. (A) Schematic overview of the STARR-seq experimental setup (adapted from [45]). (B) Cartoon representing STARR-seq peaks that were induced or repressed after activation of the Wnt/Wg pathway and the number of such peaks. (C) Distribution of induced peaks according to their fold induction. (D) UCSC genome browser screenshot of STARR-seq tracks in the *nkd* gene loci. (E) PWM logos for the Pan and Helper motif. (F) Fold induction of normalized luciferase signal for induced enhancers near *odd*, *how* and *lbe* genes. In red: wild-type sequences; in grey: sequences with mutated Pan motif. All DNA sequences are listed in S3 Table. Paired t-test: *p-value = 0.01; **p-value≤10–4. Data are shown as mean ± SD of two experiments.

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wingful reporter (S5A Fig). Furthermore, treatment with CHIR robustly induced expression of known Wg targets in *Drosophila* cells (S5B and S5C Fig).

Activation of the Wnt/Wg signaling pathway led to robust changes in enhancer activities: we identified 185 STARR-seq peaks (p-value $\leq$ 0.001) that were at least 3-fold induced in the CHIR-treated versus control sample, and 348 that were at least 3-fold repressed (Fig 6B). Among the induced peaks, 73 (39.5%) were induced more than 5-fold and 32 (17.2%) more than 10-fold (Fig 6C). We found several enhancers, which have already been described as WREs in *Drosophila* Kc cells. For instance we identified two enhancers close to the TSS of *nkd*

(first intron and 10 kb upstream of TSS) (Fig 6D), the well-studied WRE 2.2 kb upstream of the TSS (transcription start site) of *Notum*, an enhancer 15.2 kb upstream of *pxb* and an element in the 5' intergenic region 178 bp upstream of *Ugt36Bc* [50, 43, 20] (S4B Fig). We validated activated and repressed STARR-seq enhancers in luciferase reporter assays as described in [47]. Consistent with the STARR-seq results, we found luciferase reporter activities responded as expected to both CHIR treatment and WCM treatment: increased activity for activated enhancers and decreased activities for repressed enhancers (S4C and S6 Figs). Taken together, these results indicate that the activities of STARR-seq detected enhancers are modulated by Wnt/Wg signaling.

The TCF/Pan motif is necessary for Wnt/Wg-induced enhancers

To further test that the identified enhancers were directly regulated by Pan, we assessed the enrichment of known transcription factor motifs [46] in Wnt/Wg-responsive STARR-seq enhancers in comparison to negative control sequences (see Material and Methods). The known TCF/Pan motif [51] (Fig 6E) was strongly enriched in induced enhancers (2.7-fold enrichment, p-value = $1.3x10^{-8}$), whereas it was not enriched in constitutive or repressed enhancers (p-value = 0.27 and p-value = 0.08, respectively). Using *de novo* motif discovery (see Material and Methods) we found an additional Helper site motif in induced enhancers (GCCGCC, p-value = $3.4x10^{-14}$; Fig 6E), which is a GC-rich element near TCF/Pan binding sites that is critical for Wnt/Wg target gene activation [52–53, 11]. To experimentally validate the necessity of the TCF/Pan motif for Wnt/Wg induced enhancers, we tested wild-type and mutated versions of the TCF/Pan motif in 3 enhancers of the *odd*, *how* and *lbe* genes in luciferase assays. While the wild-type enhancers activated luciferase reporters 31-, 11- and 7-fold after Wnt/Wg induction by CHIR treatment, the Pan motif-mutant sequences did not respond to treatment (<1.2-fold induction), a substantial and significant difference in each case (p-value≤0.01; Fig 6F), indicating that at least these 3 Wnt/Wg-responsive enhancers require the TCF/Pan motif.

Pan regulates Wnt/Wg-responsive enhancers

Given the enrichment of the TCF/Pan motif in the Wnt/Wg-responsive STARR-seq enhancers and the necessity of this motif for enhancer function, we next examined whether Wnt/Wg-responsive enhancers require the Pan protein. We repeated the STARR-seq experiments in *pan* null mutant cells (S7A Fig) and again confirmed our findings for a subset of the enhancers by treatment with WCM (S6 Fig). Consistent with our analysis of target gene expression by RNA-seq, we found that enhancer-induction was overall strongly reduced from 26.1-fold the highest induction in wild-type cells to at most 3.8-fold in *pan* null mutant cells and that the vast majority (80%) of Wnt/Wg-induced enhancers no longer responded to pathway activation (Fig 7A). For example, the enhancers in first intron and 10 kb upstream of TSS in the *nkd* gene locus that were strongly induced in wild-type cells by Wnt/Wg signaling were not any more induced nor detected in *pan* knockout cells (p-value>0.001, Fig 7B). We confirmed these findings by testing several of the most strongly activated enhancers in luciferase reporter assays. In agreement with the STARR-seq results, enhancers that were strongly activated by Wnt/Wg signaling in wild-type cells did not respond to Wnt/Wg pathway activation in *pan* knockout cells (S7B Fig). Taken together, these results argue that Pan is required for the activation of Wnt/Wg-responsive enhancers.

Discussion

According to the generally accepted dogma the canonical Wnt signaling pathway culminates in the transcriptional induction of target genes via the beta-catenin/TCF complex. During the

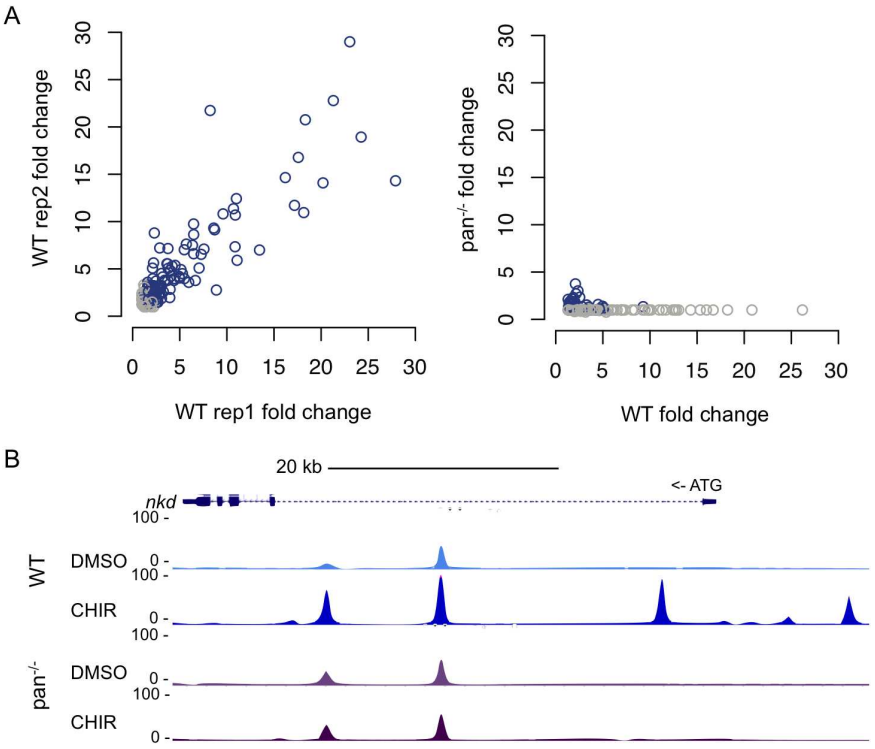

Fig 7. Wnt/Wg-induced enhancers depend on Pan. (A) Scatterplots show signal fold induction at induced enhancers for STARR-seq replicates in wild-type (WT) cells (left) and for comparison of WT versus pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells (right). Grey dots indicate non-significant induction (p-value>0.001). (B) UCSC browser screenshot of STARR-seq tracks in WT and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells for *nkd* gene locus.

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past decade, several alternative configurations of the Wnt pathway have been proposed in which either beta-catenin or TCF is bypassed. A recent study explored the co-occupancy of TCF4 and beta-catenin using ChIP-seq and showed that TCF4 is the major factor in tethering beta-catenin to DNA [24]. However, the study could not exclude the possibility that other putative factors could compensate the lack of TCF or beta-catenin–an aspect that is still poorly understood in the field of Wnt research. In the present study, we investigate whether and, if yes, to which extent a Wnt response can bypass beta-catenin or TCF. To this aim we used somatic cell genetics in *Drosophila* cultured cells. As a basis for our analysis, we first carried out a systematic and genome-wide study to explore all Wnt/Wg-related transcriptional outputs in this system. We identified a set of 51 genes that are induced upon Wg stimulation. To probe whether their expression requires Arm or Pan, we generated cells lacking one or the other of these factors using the CRISPR/Cas9 technology. Surprisingly, we found that Arm and Pan are both absolutely required for all Wnt/Wg-related transcriptional outputs in this system. As a transcription factor, Pan binds to DNA regulatory elements up- or downstream of the TSS of its target genes. Thus, next we asked, whether these DNA regulatory elements–enhancers/repressors–are dependent on Pan using STARR-seq. Impressively, consistent with our RNA-seq analysis, we found that the induction of Wnt/Wg-responsive enhancer elements fully depends on Pan.

In our work we identified eleven down-regulated target genes and showed that knockout of Arm or Pan is sufficient to abrogate their repression. We observed the same effect for

repressed enhancers in *pan* null mutant cells. These findings are in line with a previous study in *Drosophila* Kc cells [20], in which it was shown that Pan and Arm are required for the repression of the negative target genes *Pxn*, *Ugt36Bc*, *Tig* and *Ugt58Fa* [20]. We also found *Pxn* in our Wnt/Wg target gene set. However, the other genes were less than 2-fold repressed in our system and thus did not pass our selection criteria. This might be due to technical differences in Wnt-pathway stimulation and/or timing. Blauwkamp and colleagues showed also in their study that the negatively regulated targets exhibited lower expression upon Pan reduction in the Wnt OFF state [20], implicating that Pan normally activates their expression even in the absence of Wg ligand. When analyzing our data, we found that only half of the negative target genes appear to be activated in the Wnt OFF state upon Pan abrogation, the remaining targets did not exhibit a significant change in their expression profile. This suggests that they might be indirect targets or independent of Pan. Furthermore, we found that several repressed enhancers possess neither the traditional TCF/Pan binding motif, nor the previously reported alternative binding site important for repression, indicative for a Pan-dependent indirect regulation of repressed enhancers. It is likely that Pan is tethered to the DNA by other co-factors as it was shown for *dpp* or *CDH1* [21, 23]. Thus, these Pan-dependent enhancers without any known TCF/Pan binding site provide a good starting point for further molecular studies to gain insight into the still incomplete model of Wnt-mediated repression [16].

In sum our results demonstrate that all Wnt/Wg-related transcriptional output in *Drosophila* cells requires Arm and Pan and that the induction of Wnt/Wg-responsive enhancers is fully dependent on Pan. Hence, collectively our data argue against the existence of distal branching of the Wnt pathway in this system.

Materials and methods

Drosophila cell culture

Drosophila Kc167 cell lines were cultured in M3+BYPE medium, supplemented with 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 25˚C.

Activating Wnt signaling in *Drosophila* Kc cells

Wg-CM was harvested from S2 tubulin wingless cells. S2 tubulin wingless cells were seeded 24 h prior collecting the supernatant ($1x10^6$ cells/ml) by centrifuging the cells at 3500 rpm for 5 min. For the control medium S2 cells were prepared as described above. WCM or CM was added to Kc cells for 24 h to induce Wnt/Wg signaling. To induce the Wnt/Wg signaling pathway with CHIR99021 (S1263, Selleckchem), 25 μM of the inhibitor was used and added to the medium for 24h. As control DMSO was used. After 24 h of induction, cells were harvested.

Cas9 and gRNA plasmids

Cas9 (49330, Addgene) and empty gRNA vector (49410, Addgene) were obtained from Addgene. Oligo design and cloning was accomplished after manufacturer's protocol.

Mutagenesis of genes with CRISPR/Cas9

CRISPR was performed as described in [37]. Briefly, cells were plated at 2 x 10^6 cells per well of a 6-well dish and a total of 1.7 μg DNA, Cas9 and gRNA in a 1:1 ratio, was co-transfected into each well using Fugene HD (Promega) at a 1:2 ratio (μg:μl), following manufacturer's instructions. Both gene loci were targeted simultaneously using a gRNA and Cas9 with integrated gRNA. Transfections were analyzed after 3 days, and selection was performed in 5 μg/ml Puromycin (P8833 Sigma). The genotype was analyzed using PCR primers spanning the cut site.

PCR products were cloned in pGEMT-vector system (Promega) and 10–100 clones were analyzed by sequencing. Primers for gRNA cloning and for detection of CRISPR events are available in the S1 Table.

Western blot

Nuclear protein extraction was performed as described in [54]. For Western blot analysis, monoclonal anti-Arm (1:500; N2(7A1), DSHB) and monoclonal anti-alpha-Tubulin (1:5000; T5168, Sigma) antibodies were used and followed by HRP-anti-mouse IgG (705-035-003, Jackson Immuno Research Laboraties, inc).

qRT-PCR

Real-time q-PCR analyses were carried out with SYBR Green Supermix (BioRad) on a iCycler iQ real-time OCR detection system (BioRad). For qRT-PCR, total RNA was extracted from 1–2 x 10^6 cells with NucleoSpin RNA extraction kit from Macherey-Nagel according to the manufacture's protocol and reverse transcribed with Roche, followed by qRT-PCR. Sequences of the primer pairs used are listed in S1 Table.

RNA-seq

All pair-end sequencing was performed on an Illumina HiSeq2500 machine at the Genomics Platform of the University of Geneva. For all experiments we compared three independent biological replicates and merged them for the subsequent analysis. All RNA-seq files are available from SRA NCBI database. Submission code: SUB2472808; Study: PRJNA378604 (Accession Number SRP101692).

Computational analysis

All deep-sequencing data were mapped to the *Drosophila* reference genome dm3 using TopHat and analyzed as described in [34] and using thresholds as indicated above. We used GraphPad Prism for all statistical analysis and R for plotting.

STARR-seq

STARR-seq in *Drosophila* WT cells and *pan* knockout cells was performed in two biological replicates as described in [47]. To obtain Wnt-responsive enhancers, cells were treated with 25μM CHIR99021 or DMSO for 24h. Data were analyzed as described in [47]. For Fig 7A fold enrichments were calculated directly over DMSO-treated samples at summits of induced enhancers and p-values indicate significance of the fold change. All STARR-seq files are available at the GEO database (GEO number GSE96542).

Motif analysis

For TCF/Pan motif enrichment analysis, we used 200 bp regions around the summit of 185 induced, 348 repressed, 1834 constitutive enhancers, and 987 random sequences that were not detected with STARR-seq but followed the same genomic distribution (denoted as negative regions). Enrichments were calculated as described [46]. *De novo* motif analysis was done with DREME using negative regions as a background set (see S2 Table).

Reporter assay

Enhancer candidates were amplified from genomic DNA of *Drosophila* Kc167 cells (for primers see S3 Table). All candidates were subcloned to either pCR8/GW/TOPO (Invitrogen) or pENTR/TOPO (Invitrogen) and delivered into the firefly luciferase vector [45] using the Gateway LR Clonase II enzyme mix (Invitrogen). Kc cells ($1x10^5$) were transfected using Fugene HD (Promega) with a total of 300 ng of various plasmid combinations (1:3 ratio of promoter reporter plasmid to Renilla). Luciferase activities were measured 48 h after transfection and after stimulation with either Wg ligand or CHIR99012 using the Dual-Luciferase Reporter Assay System (Promega). Every experiment was repeated at least twice with three replicates in each independent experiment. Enhancers' sequences used are listed in S3 Table.

Supporting information

S1 Fig. (A) Schematic representation of potential protein products of Arm in $arm^{-/-AFII7/8}$ ($arm^{-/-}$) cells with premature termination codons (stop), which result from introduced frameshift mutations. (B) Full Western blot analysis from Fig 2. As presented in the blot, no truncated versions of Arm could be detected.
(TIF)

S2 Fig. qRT-PCR analysis of (A) positive and (B) negative candidate Wnt/Wg target genes in wild-type (WT), $arm^{-/-AFII7/8}$ ($arm^{-/-}$) and $pan^{-/-AF1AD26}$ ($pan^{-/-}$) cells. Cells were stimulated with WCM or CM for 24 h. Analysis of expression profiles of several Wg target genes after treatment versus control confirmed their induction after WCM stimulation. Fold expression changes of mRNA were calculated by dividing WCM treatment-driven expression values by the expression values obtained with the control treatment.
(TIF)

S3 Fig. (A) Schematic representation of potential protein products of Pan in $pan^{-/-AF1AD26}$ ($pan^{-/-}$) cells with premature termination codons (stop) due to introduced frameshift mutations. (B) qRT-PCR analysis of *pan* mRNA level with primer targeting its N-term (see S1 Table) in wild-type (WT) and $pan^{-/-AF1AD26}$ ($pan^{-/-}$) cells. Cells were stimulated with WCM or CM for 24 h. Fold expression changes of mRNA were calculated by dividing WCM treatment-driven expression values by the expression values obtained with the control treatment.
(TIF)

S4 Fig. (A) Scatterplots of replicates of STARR-seq in wild-type (WT) cells treated with DMSO or CHIR99021 (CHIR). (B) UCSC browser screenshot of STARR-seq tracks in WT cells for *pxb*. (C) Validation of peaks from the constitutive, induced, and repressed enhancer classes by luciferase assays. Log2 fold induction (CHIR-treated versus control) of normalized luciferase signal is shown. Wilcoxon rank-sum test: **p-value = 0.0007, *p-value = 0.003, n indicates the number of enhancers in each group.
(TIF)

S5 Fig. CHIR99021 activates reliably and efficiently Wnt/Wg target genes in Drosophila cells. (A) Titration of CHIR99021 in *Drosophila* S2R+ cells. S2R+ cells were transfected with *wingful* luciferase reporter vector and Renilla. Red bars: promoter activation with 25 μM CHIR is as efficient as with Wg ligand. In green is the *wingful* promoter activity after stimulation with ArmS10 depicted, black bar shows the activity after control treatment, grey bars represent the activity after respective CHIR99021 concentration. (B, C) qRT-PCR analysis of gene expression in *Drosophila* Kc cells in the Wnt OFF and ON state. Fold change of gene expression levels were calculated using expression values after WCM (A) or CHIR (B) treatment

versus control treatments. Stimulation with WCM and CHIR leads to a similar robust expression of target genes *nkd*, *fz3* and *Toll-7* in wild-type cells.
(TIF)

S6 Fig. Validation of (A) induced and (B) repressed candidate STARR-seq enhancers with WCM. Candidate enhancer sequences were cloned into the STARR-seq library luciferase vector, see Material and Methods. Wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells were transfected with the candidate luciferase reporter expression vector and Renilla expression vector 24 h prior stimulation with WCM (as control CM was used). After 24h stimulation, reporter activity was analyzed.
(TIF)

S7 Fig. (A) Scatterplots of replicates of STARR-seq in pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells treated with DMSO or CHIR99021 (CHIR). (B) Validation of candidate STARR-seq enhancers. Candidate enhancer sequences were cloned into the STARR-seq library luciferase vector, see Material and Methods. Wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells were transfected with the candidate luciferase reporter expression vector and Renilla expression vector 24 h prior stimulation with CHIR (as control DMSO was used). After 24h stimulation, reporter activity was analyzed.
(TIF)

S1 Table. Primer sequences for qRT-PCR, cloning of gRNAs, PCR.
(XLSX)

S2 Table. *de novo* motif search using DREME.
(XLSX)

S3 Table. Primer sequences for STARR-seq enhancer validations and Pan motif validation.
(XLSX)

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Writing – review & editing: AF DS EB AS KB.

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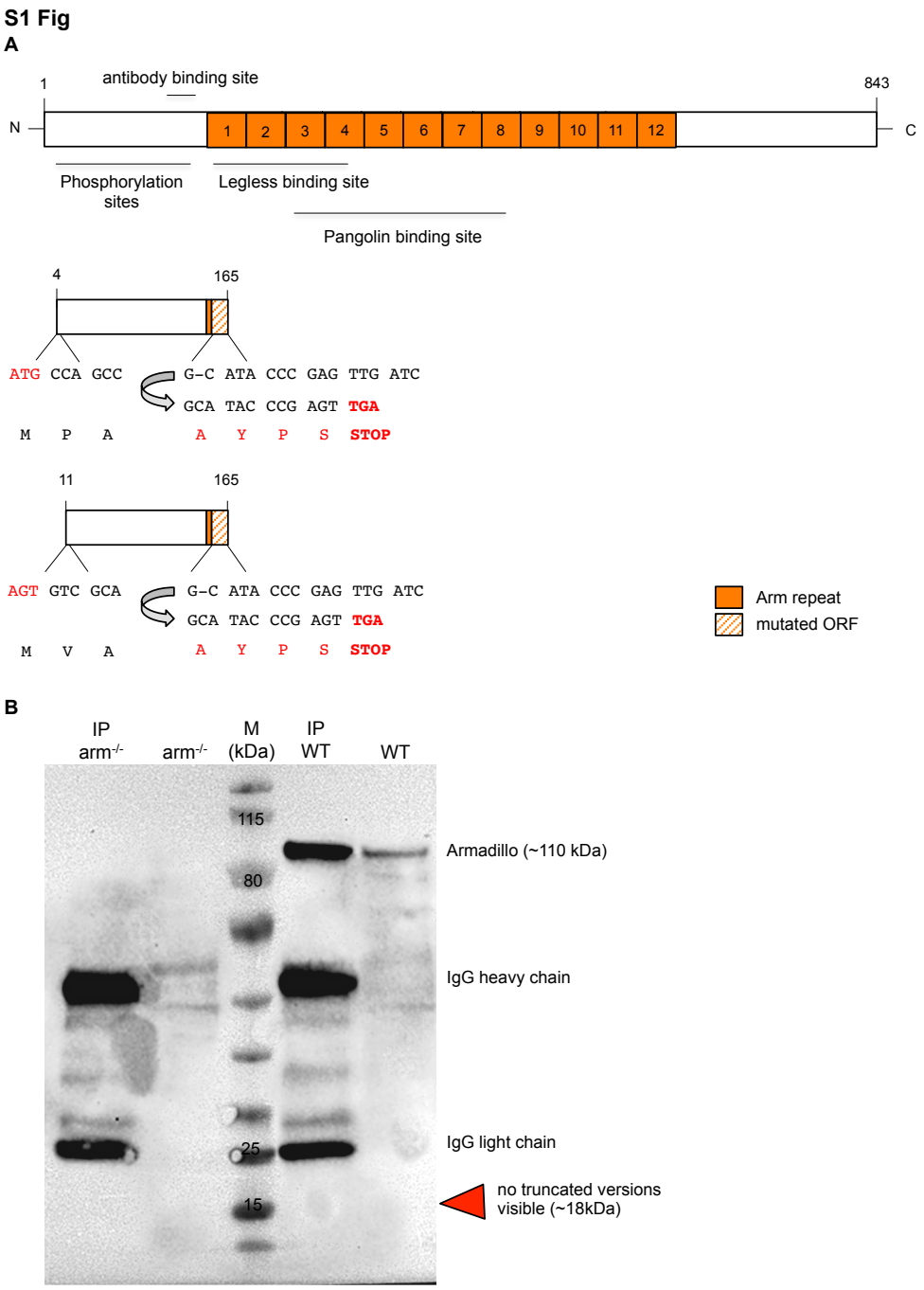
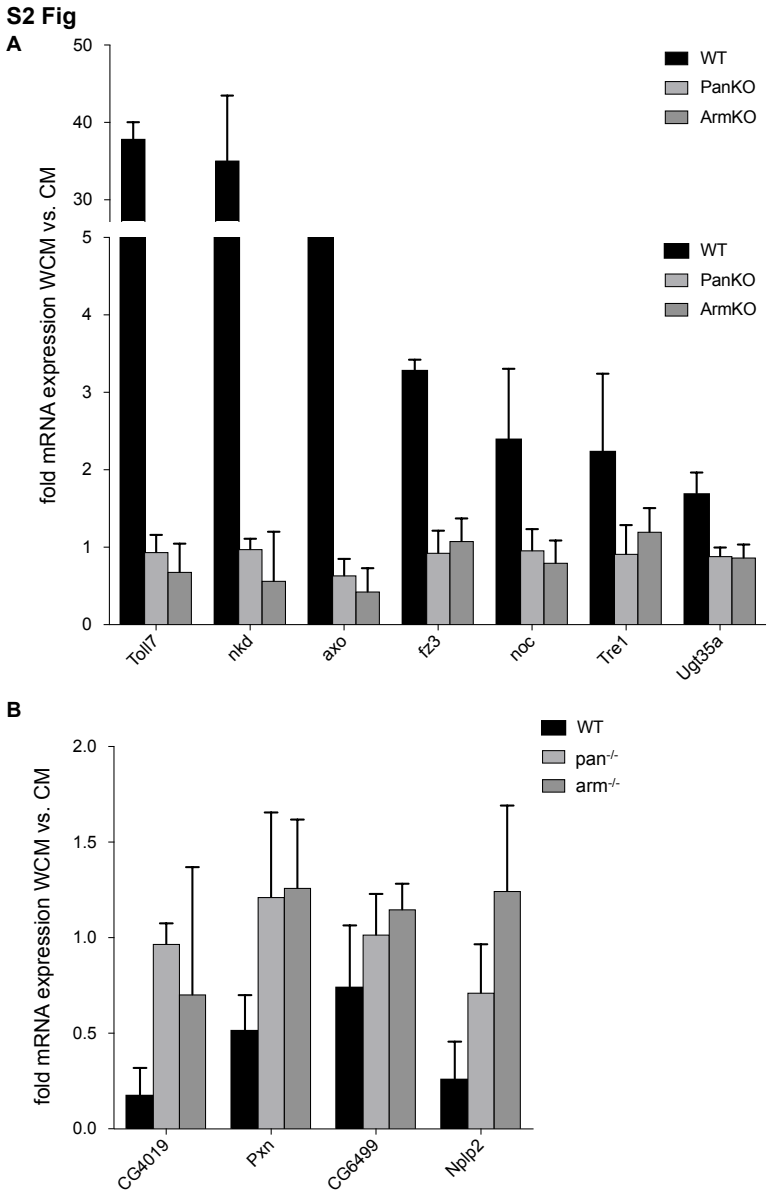
Supplementary figures and figure legends for "Probing the canonicity of the Wnt/Wingless signaling pathway"

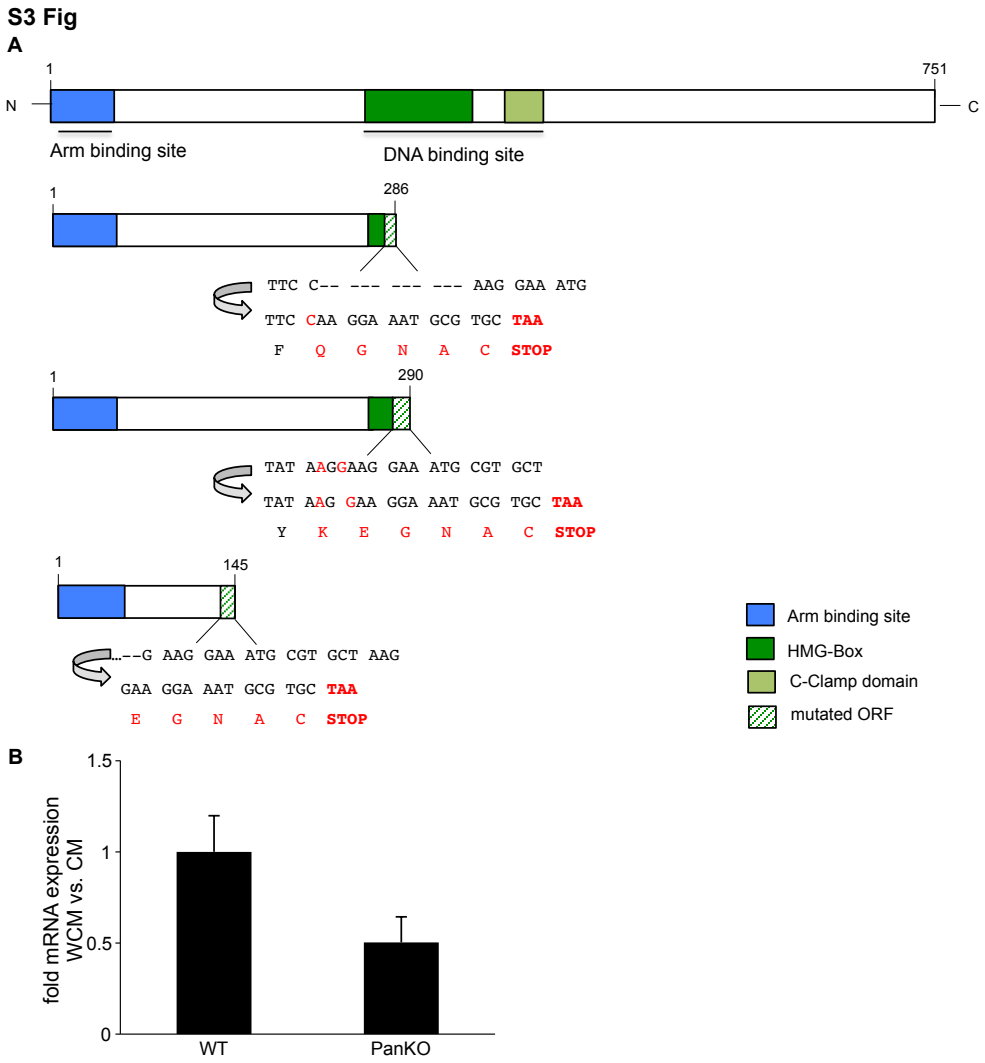
S1 Fig



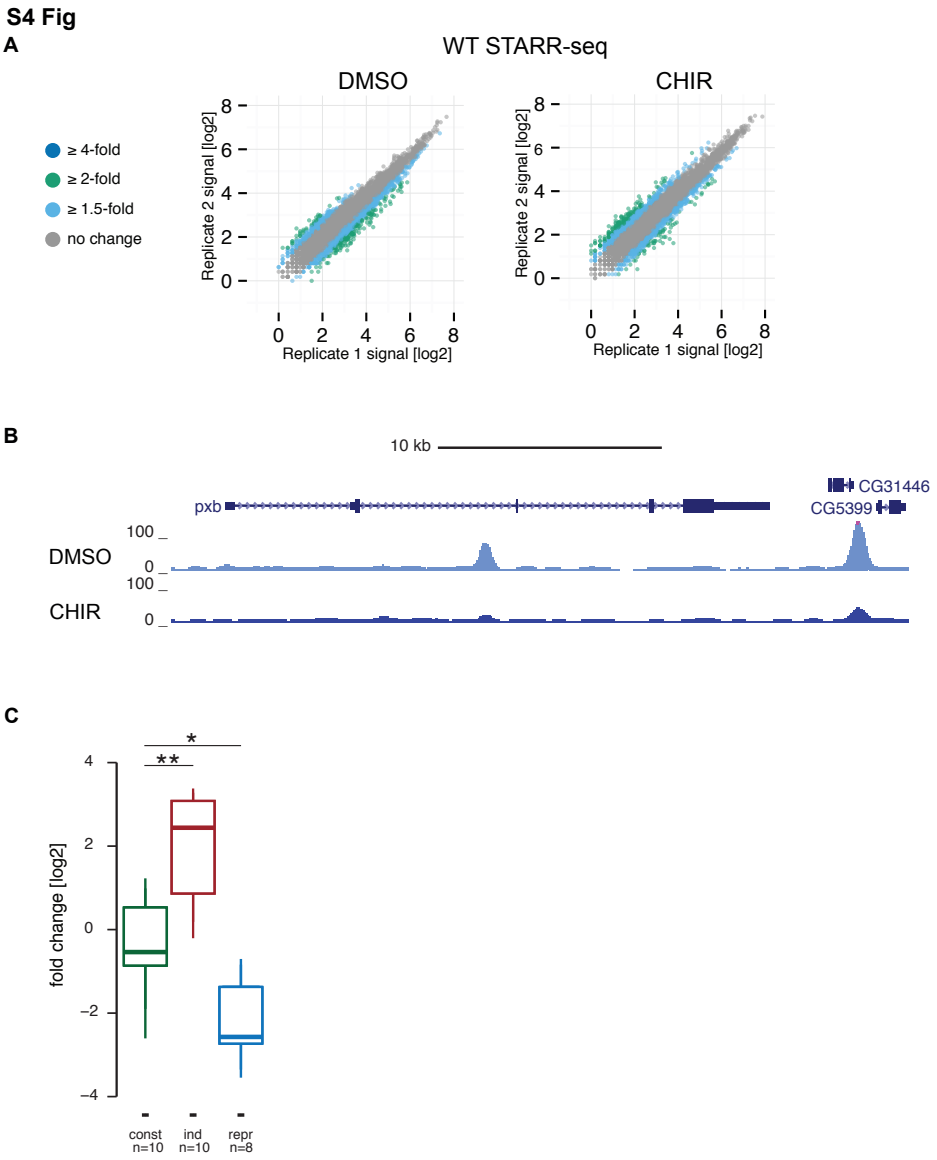
S1Figure (A) Schematic representation of potential protein products of Arm in arm$^{-/-AFII7/8}$ cells with premature termination codons (stop), which result from introduced frameshift mutations. (B) Full Western blot analysis from Fig 2. As presented in the blot, no truncated versions of Arm could be detected.



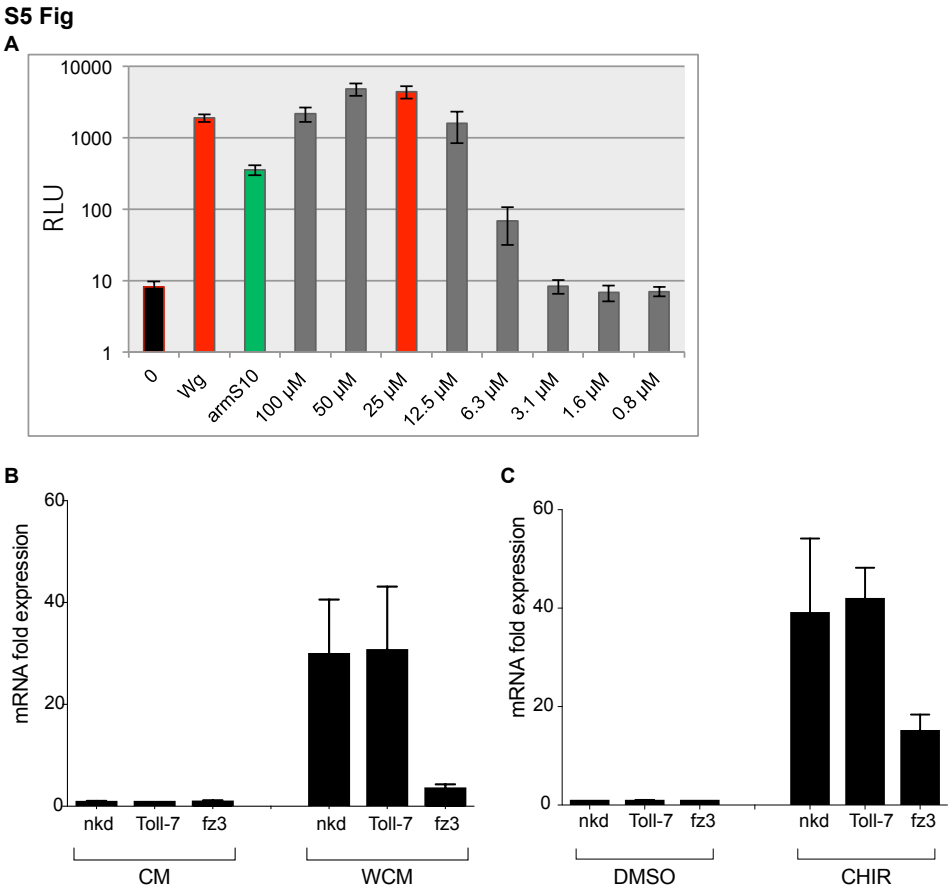
S2Figure qRT-PCR analysis of (A) positive and (B) negative candidate Wnt/Wg target genes in wild-type (WT), $arm^{-/-AFII7/8}$ ($arm^{-/-}$) and $pan^{-/-AF1AD26}$ ($pan^{-/-}$) cells. Cells were stimulated with WCM or CM for 24 h. Analysis of expression profiles of several Wg target genes after treatment versus control confirmed their induction after WCM stimulation. Fold expression changes of mRNA were calculated by dividing WCM treatment-driven expression values by the expression values obtained with the control treatment.



S3Figure (A) Schematic representation of potential protein products of Pan in pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells with premature termination codons (stop) due to introduced frameshift mutations. (B) qRT-PCR analysis of pan mRNA level with primer targeting its N-term (see S1 Table) in wild-type (WT) and pan$^{-/-AF1AD26}$ (pan$^{-/-}$) cells. Cells were stimulated with WCM or CM for 24 h. Fold expression changes of mRNA were calculated by dividing WCM treatment-driven expression values by the expression values obtained with the control treatment.



S4Figure (A) Scatterplots of replicates of STARR-seq in wild-type (WT) cells treated with DMSO or CHIR99021 (CHIR). (B) UCSC browser screenshot of STARR-seq tracks in WT cells for pxb. (C) Validation of peaks from the constitutive, induced, and repressed enhancer classes by luciferase assays. Log2 fold induction (CHIR-treated versus control) of normalized luciferase signal is shown. Wilcoxon rank-sum test: **p-value = 0.0007, *p-value = 0.003, n indicates the number of enhancers in each group.



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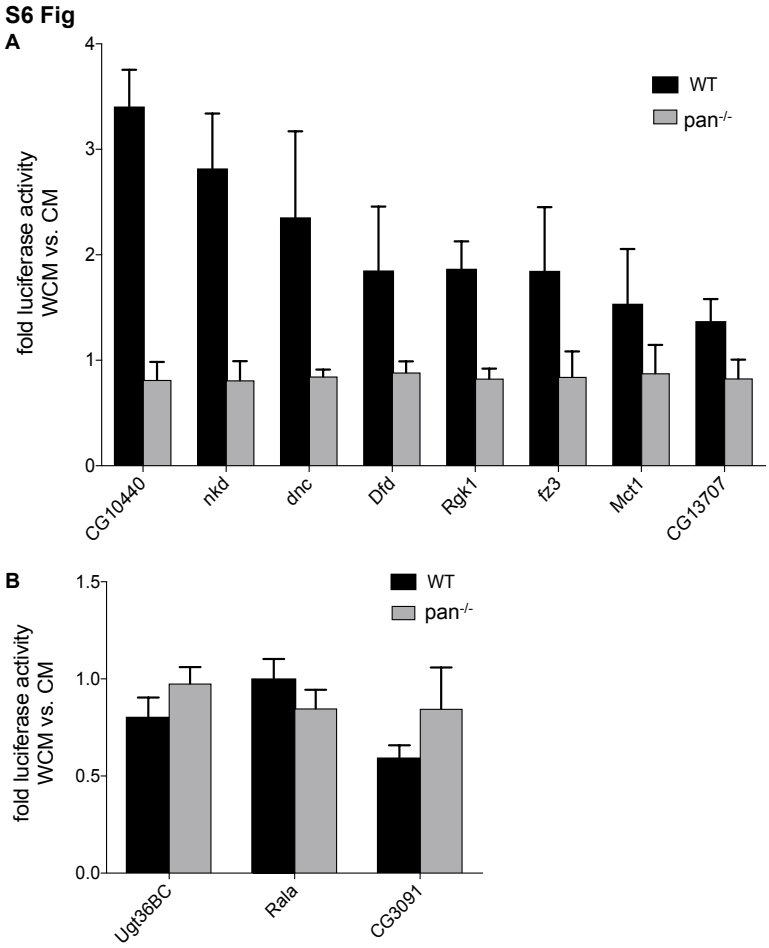

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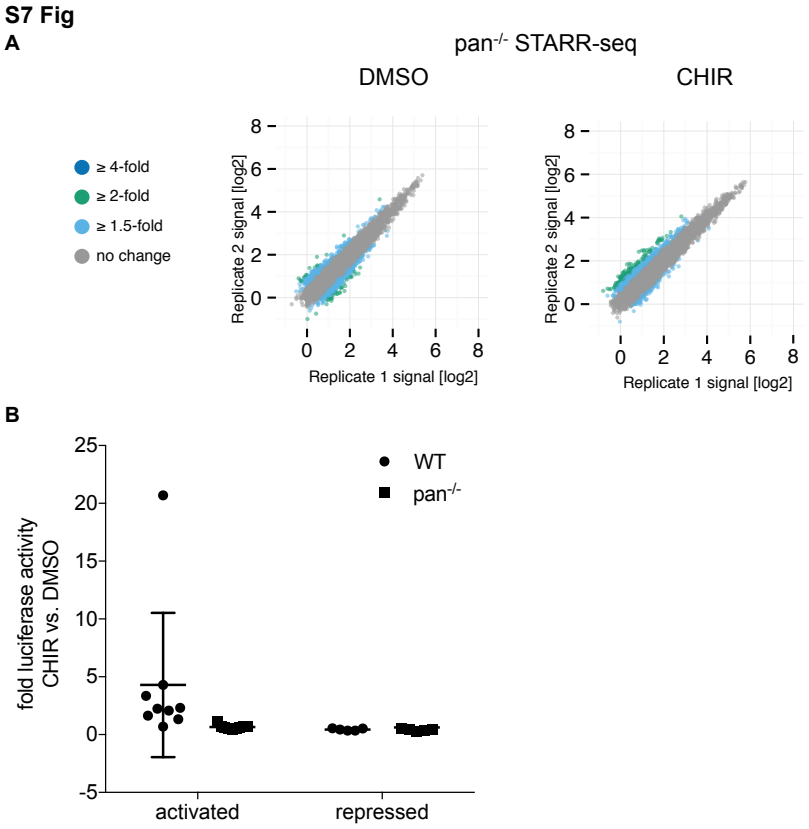

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3.1.1 Generation of genome-modified *Drosophila* cell lines using SwAP

This chapter describes a highly efficient workflow for generating Cas9-modified *Drosophila* cell lines. The workflow overcomes many of the problems that plague single cell cloning with *Drosophila* cells.

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Generation of genome-modified *Drosophila* cell lines using SwAP

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Abbreviations: Arm, Armadillo; AS, Allele-Specific; CRISPR, Clustered Regularly Interspaced Short Palindromic Repeats; DGRC, Drosophila Genomics Resource Center; NHEJ, Non-Homologous End Joining; SwAP, pre-Selection with Allele-specific Primers

Abstract

The ease of generating genetically modified animals and cell lines has been markedly increased by the recent development of the versatile CRISPR/Cas9 tool. However, while the isolation of isogenic cell populations is usually straightforward for mammalian cell lines, the generation of clonal *Drosophila* cell lines has remained a longstanding challenge, hampered by the difficulty of getting *Drosophila* cells to grow at low densities. Here, we describe a highly efficient workflow to generate clonal Cas9-engineered *Drosophila* cell lines using a combination of cell pools, limiting dilution in conditioned medium and PCR with allele-specific primers, enabling the efficient selection of a clonal cell line with a suitable mutation profile. We validate the protocol by documenting the isolation, selection and verification of eight independently Cas9-edited *armadillo* mutant *Drosophila* cell lines. Our method provides a powerful and simple workflow that improves the utility of *Drosophila* cells for genetic studies with CRISPR/Cas9.

Introduction

The discovery and adaptation of the clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 system and its application in diverse species, including yeast,[1] fruit fly,[2-7] zebrafish,[8-10] mouse,[11-13] and human cells [14,15] has reshaped the landscape of molecular biology. Today, scientists are able to easily and efficiently engineer virtually any genome at specific loci.[16-18] The Cas9 protein is an RNA-guided DNA endonuclease recognizing a short trinucleotide NGG protospacer motif sequence (PAM) adjacent to the cognate target sequence.[19,20] Subsequent Cas9 cleavage of the double-stranded DNA is followed by DNA repair events. The introduced double-strand breaks are mended either by non-homologous end joining (NHEJ) potentially leading to mutational events in the target site or by homology directed repair via a donor template.[21,22]

The CRISPR/Cas9 system has also been implemented in cultured *Drosophila* cell lines.[23,24] *Drosophila* cell lines are an widely used experimental system, complementing the insights into basic biological mechanisms, genes functions, and disease obtained in flies (for a review, see ref. 25). The advantages of fly cell culture over mammalian cells are of technical and biological nature, such as their high susceptibility to RNAi and their simple genomic structure with less redundancy, providing a powerful gene discovery tool.[26,27] Currently, more than 150 fly cell lines are publicly available from the Drosophila Genomics Resource Center (DGRC)

among which, S2, Clone-8 and Kc167 are the most commonly used ones. These lines have also been used for large-scale studies such as the modENCODE project investigating genomic structural elements.[28]

The applications of the CRISPR/Cas9 system in *Drosophila* tissue culture ranges from its general applications of generating genetic mutations,[23,24,29] to CRISPR interference studies [30,31] and the establishment of a genome-wide CRISPR library for high-throughput screens.[32] However, one historical challenge when working with *Drosophila* cells is their difficulty to grow at low densities probably as they require essential growth stimulating factors secreted from neighboring cells.[33] This problem impedes the generation of clonal genetically modified *Drosophila* cell lines vitiating potential advantages gained by implementing targeted genome editing technologies such as CRISPR/Cas9. Several methods for cloning have been reported such as cloning by limiting dilution in conditioned medium,[34] irradiated feeder layer cells or soft agar plates (for a review, see ref. 33). However these methods are not widely used because of the low cloning efficiencies and the significant amount of time and work needed to isolate clonal lines, especially when no selectable markers (e. g. fluorescence, drug resistance) are used to isolate the clone of interest. Indeed, to our knowledge in addition to our previous study, only another research article has reported the successful generation of isogenic Cas9-engineered *Drosophila* cell lines.[31,35]

Following up on our initial publication, here we describe in detail an efficient workflow that overcomes the impediments to isolating clonal, CRISPRed *Drosophila* cell lines. We have developed a selection protocol, named SwAP (pre-Selection with Allele-specific Primers) that enables *Drosophila* researchers to efficiently identify, isolate and discriminate Cas9-engineered *Drosophila* cell clones. Our method is based on combining (i) the speed and scale of cell pools to first determine pools of cells carrying a CRISPR induced modification (or combination thereof) of interest by sequencing, (ii) from this simplified population limiting dilution in conditioned medium is used for cell cloning and (iii) allele-specific (AS) primers are used to easily identify the clone of interest, which can then be expanded (Fig 1).[36-38] Using this approach, researchers can efficiently determine the allelic status and then isolate clonal cell lines with suitable mutation profiles in little time.

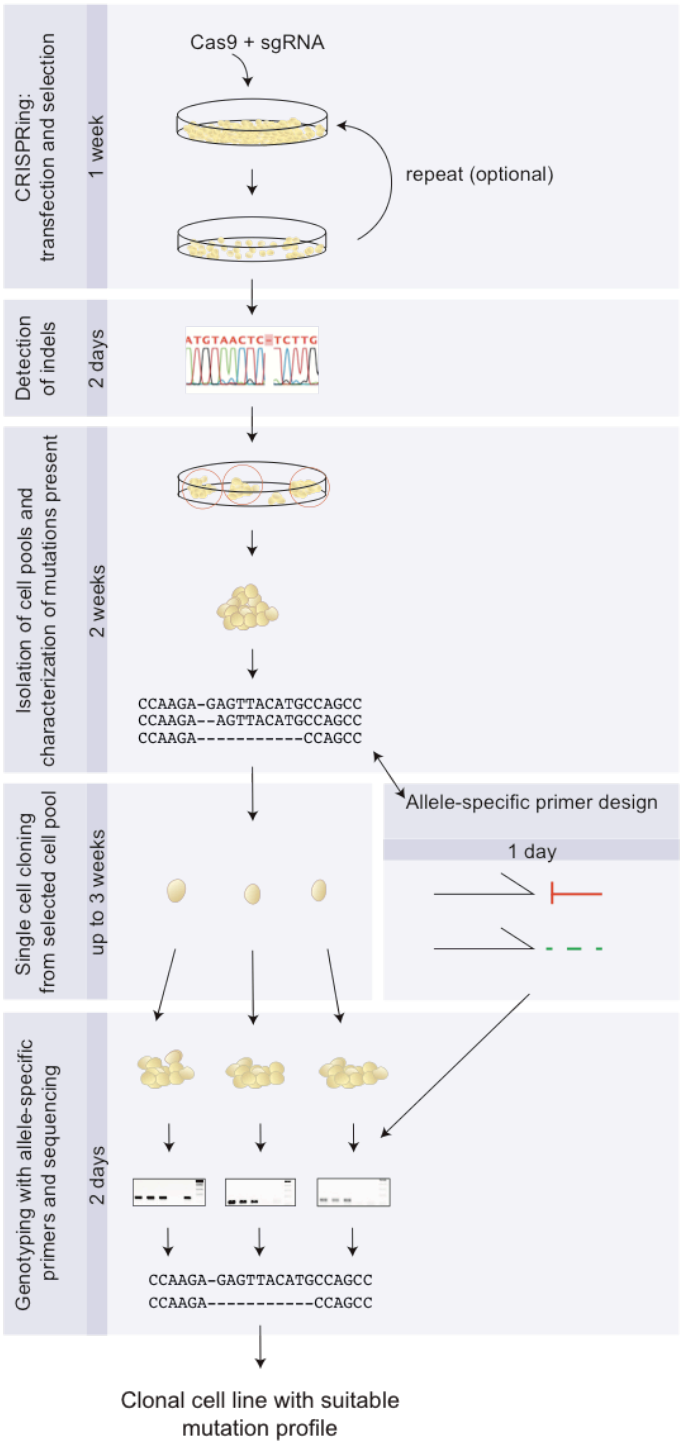

Figure 1. Overview of the workflow for the selection and identification of clonal Cas9-engineered *Drosophila* cell lines. Steps with timelines for CRISPRing cells, detection of indels using PCR and Sanger sequencing, isolation and characterization of cell pools, single cell cloning and genotyping using AS-primers and Sanger sequencing are schematically depicted. Transfected cells are selected in puromycin for 5 days and optionally reCRISPRed if a poor efficiency is observed (6 days). Selected cells are assayed for Cas9-mediated genomic modifications using PCR and sequencing (4 days). In the next step, individual cell

pools are isolated and cultivated (~ 2 weeks) and their genetic modifications are examined to pre-select efficiently favorable CRISPRed cells for single cell cloning (4 days). The sequencing results are also used to design AS-primers to screen single cell clones for desired mutation (1 day). In the next step, single cells from the selected cell pool are isolated and expanded using limiting dilution in conditioned medium (~ 4 weeks). In the last step AS-primers specifically targeting the desired mutation are used for genotyping (1 day).

Other common genotyping approaches used in conjunction with the CRISPR/Cas9 system, including the Surveyor assay (Cel1), T7 endonuclease 1 (for review, see ref 39), HRMA [40] and PAGE, [41] do not provide this level of detail. Here, we illustrate the general applicability of our approach by describing the generation of clonal *armadillo* (*arm*) mutant cell lines. Our protocol does not only greatly reduce the time and work requirements for generating clonal genome-engineered *Drosophila* cells but also meets the demand for an efficient cell cloning and selection strategy in the era of CRISPR/Cas9.

Results and Discussion

CRISPRing *Drosophila* cells

Our goal was to devise a strategy that mitigates the difficult challenge of generating isogenic CRISPRed *Drosophila* cells lines. To develop such a protocol, we used *Drosophila* Kc167 cells from DGRC; they are derived from embryonic hemocytes lineages and are pseudo diploid.[42] We choose to generate additional clonal lines in which we had genomically engineered the *arm* gene.[31] We applied the same CRISPR strategy used in our recent publication to abrogate the function of *armadillo* (*arm*) with CRISPR/Cas9: two independent sgRNAs targeting the *arm* locus (Fig 2A). We transfected the cells simultaneously with two pAc-sgRNA-Cas9 expression vectors, each harboring one gRNA expression unit that targeted either the second or the third *arm* exon (Fig 2A). The vector also contains the puromycin resistance gene as a selection marker.[23] Transfected cells were selected 24 h after transfection in medium containing 5µg/ml puromycin. To avoid randomized, stable integration of the expression vector, selective pressure was stopped after 5 days.

Determination of the spectrum of genomic modifications

Having CRISPRed the cells we next wanted to identify the types of modifications that have been generated. We therefore analyzed the gRNA target sites by PCR and Sanger sequencing to determine whether the targeted locus exhibits a big deletion of 2700 bp that had been created by simultaneous cut events at both sites or if only

smaller indel mutations at each individual target site had been generated. We collected an aliquot of approximately 1000 cells of our transfected cell population, extracted genomic DNA and first determined by PCR whether a big deletion has been generated; we used primers flanking the cut sites (Fig 2B). The PCR results revealed that within our CRISPRed cell population some cells indeed harbored, at least, one *arm* allele resulting from the big deletion: an amplicon of around 550 bp was detected which would be expected if a deletion of 2700 bp had occurred. Next, we amplified each targeted site by PCR with primers spanning the individual cleavage sites to detect whether single cuts were present. To this end, we isolated the PCR products and cloned them into the pGEMT-vector (Promega). The pGEMT-system allows efficient cloning of PCR products as the linearized vector provides compatible 3´-T overhangs at the insertion site for PCR products with polymerase-added desoxyadenosine (see Material and Methods). We sequenced DNA from 10 independent colonies for each target site and were able to detect various indel mutations at the predicted cleavage locus. Analysis of the sequences revealed that 50% of sequenced alleles had indel mutations in the second exon and 57% of alleles showed mutational events at target site two (Fig 2B). As at least 50% of the alleles present were still wildtype at both target sites, we re-CRISPRed the cell population once more using the same setup to obtain a higher proportion of mutated alleles.

Isolation of pools or clumps of *Drosophila* cells carrying modified *arm* alleles

The process of *Drosophila* single cell cloning is very time and work intensive with poor cloning efficiencies. We therefore first pre-selected cell populations with cells carrying genetic modifications of interest. Suitable cell pools would then be processed further. Like *Drosophila* S2 cells, Kc167 cells will start to grow in clumps at a density greater than 10^7 cells/ml,[43] which can be easily harvested and grow quickly in comparison to single cells. We hypothesized that these cell clusters (hereafter referred to as cell pools), can be exploited to identify whether some cells of a cell pool would be favorable for single cell cloning by analyzing the mutagenic events at the *arm* locus in the respective cell pool. This step may also be of great interest for researchers that look for specific genomic variations such as frameshift mutations. In addition, by identifying the genomic modifications we could design AS-primers to facilitate selection of the clone of interest (see below).

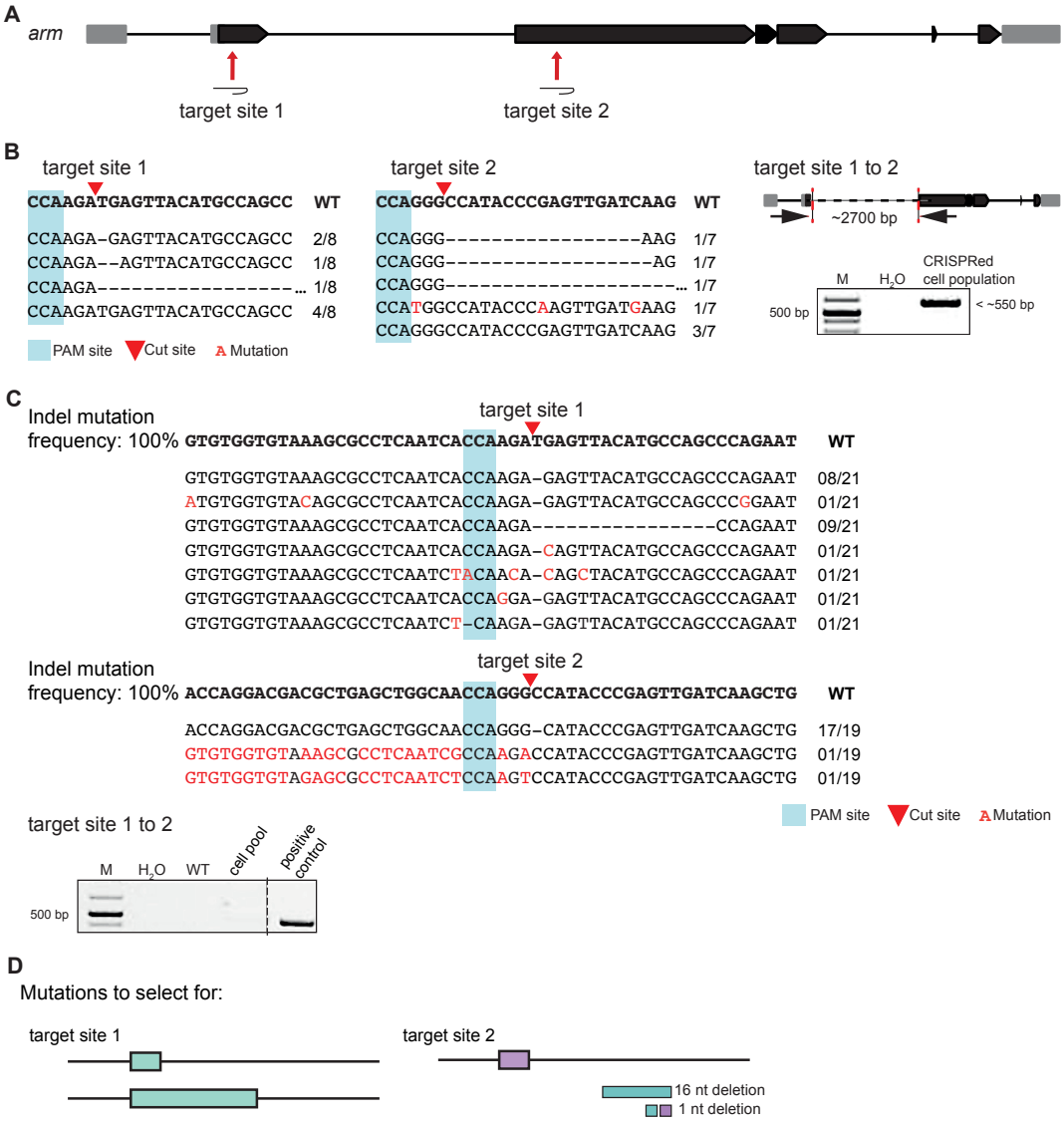

Figure 2. Detected mutations in the *armadillo* gene. (A) Schematic diagram of the *arm* locus and position of the target site 1 and 2 (red arrow). Untranslated regions (UTR) are indicated in grey boxes, translated exons in black. (B) Sequencing of indel mutations at target site one and two after transfection. PCR products spanning the cleavage site are cloned and sequenced from CRISPRed cells. The first line represents the wildtype sequence (bold). The PAM site is highlighted in blue. Schematic representation of the big deletion of 2700 bp and PCR results from primers flanking target site 1 and 2. H_2O served as control. (C) Mutational events in the analyzed cell pool. PCR produces spanning the targeted site one and two are sub-cloned and sequenced. First line represents the wildtype sequence (bold). All examined clones show indel mutations. The PAM site is highlighted in blue. PCR results detecting the big deletion of 2700 bp from wildtype (WT), water (H_2O) and cell pool. (D) Schematic representation of mutations selected clones for.

We picked 10 individual cell pools from our double CRISPRed cell population using a 1 µl-pipette and cultivated them in 50 µl of 50% conditioned and 50% fresh medium supplemented with 10% FBS in a 96 well plate. We harvested conditioned medium from confluent cells by removing the cells using centrifugation (see Material and Methods). After around 10 days, when the cells were about 50% confluent, we transferred the individual cell pools into a bigger well. As described above we determined the spectrum of mutations at the *arm* locus that are present in the cell pools. The results below describe the characterization of one such pool. We first established whether the isolated cell pool carries the big deletion of 2700 bp by PCR. In the first cell pool we analyzed, no big deletion was present since no corresponding PCR product could be amplified (Fig 2C). Next, we searched for the presence of mutational events at each cutting site by PCR using primers spanning the cut site and subsequent sub-cloning of the PCR products into the pGEMT-vector. We sequenced approximately 20 sub-cloned colonies for each site to gain a representative view of the genomic variation in the cell pool. The sequencing results revealed seven different types of mutations at the first target site (Fig 2C). The majority of sequences possessed either a deletion of one nucleotide (38%) or a deletion of 16 nucleotides (42%). Analysis of the sequencing results from the second target site showed that 89% of the alleles had a single nucleotide deletion (Fig 2C). Critically, no wildtype alleles were detected. As most of the detected alleles had either a deletion of one or 16 nucleotides at target site one (38% and 42%) and almost all alleles a one-nucleotide deletion at target site two (89%), we reasoned that these mutations would be suitable to select single clones for using AS-PCR (Fig 2D). We used the cells from the examined cell pool for single cell cloning. Moreover, the mutations we select for would in combination lead to a loss of Arm function.

Cell cloning

Several cloning protocols for *Drosophila* cells have been proposed (for a review, see ref 33). Best cloning efficiencies have been achieved for *Drosophila* Kc167 cells by dilution in conditioned medium.[44] Hence, we plated approximately 144 single cells to be cloned from the above described cell pool by limiting dilution in 50 µl 50% conditioned and 50% fresh medium, supplemented with 10% FBS in 96 well plates (Material and Methods).[34] After around 20 days, we could observe small cell colonies covering some of the wells. Once a clone covered half of the well, we transferred the cells to larger volumes and plates. We obtained cell cloning efficiencies of around 24%, permitting the expansion of 35 clones, of which 8 (11.5%) clones could be stably cultivated. Similar cloning efficiencies (~5%) have been reported from other

laboratories.[34] As soon as cell populations were stably established we identified the clones for the desired mutations by using AS-primers (see below).

Designing allele-specific primers

Allele-specific primers allow the detection of single nucleotide polymorphism (SNP) as they have the 3'-end complementary to the SNP site.[37] We wanted to select clones possessing either the deletion of one or 16 nucleotides at the first target site and the one nucleotide deletion at the target site two at the *arm* locus (Fig 2D), we designed AS-primers based on the desired sequences according to the criteria of ref. 38 (see Material and Methods and SFig1). As described in from Liu and colleagues, we modified the three bases closest to the 3'end of the forward primer, as these are essential for primer specificity.[38] No PCR product will be generated, if at least two mismatch base pairs are present within the third bases closest to the 3'end. By contrast, a PCR product will arise, if only one mismatch occurs at the 3'end. According to the same principals, we also designed primers specific for targeting the corresponding wildtype sequences as control (see Material and Methods and SFig1).

Genotyping cells using allele-specific primers

So far many genotyping approaches have been described for genome-modified animals and human cells generated by CRISPR/Cas9, such as the commonly used surveyor assay and HRMA (for a review, see ref. 39). Although the approaches can precisely detect, whether genomic modification events have occurred at the cleavage site, they do not provide information about the type of genomic modification. Hence, to identify and select efficiently a clonal cell line with a suitable mutations profile, we used AS-primers for genotyping. *Drosophila* Kc167 cells are pseudo diploid,[42] therefore isolated and expanded clones derived from the analyzed cell pool are expected to be either homozygous or heterozygous for the selected mutations at target site one. It is important to note that *Drosophila* cell lines may vary in their karyotype status, such as for instance S2 cells having a tetraploid karyotype.[45] In case cell line other than Kc167 are used; the selection and screening criteria need be adapted accordingly. Using AS-primer specifically detecting the deletion of one nucleotide at target site one, we observed a PCR product from all clones (Fig 3A), whereas no PCR was found from the AS-primer specific for the wildtype primer binding site (Fig 3A), indicating that all isolated clones harbor this deletion. Using AS-primers specific for the deletion of 16 nucleotides deletion we again observed a product in all clones (Fig 3B). As expected if the other allele harbored only the single nucleotide deletion and was wildtype at the primer binding site we detected a product

using the respective wildtype primer (Fig 3B). At target site two the isolated clones were homozygous mutant for the single nucleotide deletion (Fig 3C). Based on the above results, we conclude that generated cell clones are heterozygous for the mutations at target site one and homozygous for mutations at target site two (Fig 3D and SFig2). Most importantly, they do not harbor any wildtype allele clearly indicating a complete loss-of function of *arm* gene function. As most widely used *Drosophila* cell lines, could carry copy number variations,[46,47] as next step targeted high-throughput sequencing methods could be applied to fully characterize the ploidy state of a generated cell clone. This step might be especially useful, when working with *Drosophila* cell lines with a non-diploid karyotype status.

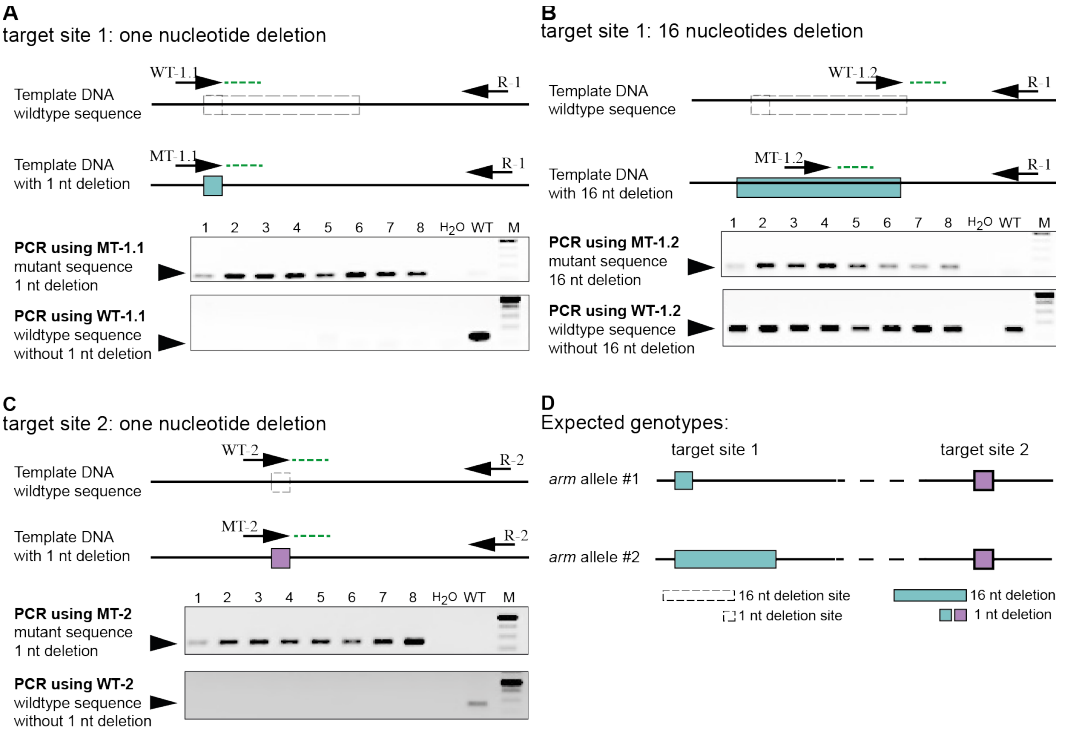

Figure 3. Schematic of AS-PCR reaction and genotyping results using AS-primers. DNA samples from eight individual *arm* mutant clonal cell lines (1-8) and from wildtype cells (WT) are genotyped using a standard PCR reaction. H_2O served as control. Arrows symbolize primers; boxes with dotted lines represent the deletion site, boxes with solid lines represent the deletion. Green dotted lines represent the ability of the AS-primer to bind. (A) AS-PCR for the deletion of one nucleotide or the corresponding wildtype sequence at target site one. PCR is performed with primers targeting one nucleotide deletion (MT-1.1) or the corresponding wildtype sequence (WT-1.1). For all PCR reactions a common reverse primer is used (R-1). (B) AS-PCR for the deletion of 16 nucleotides at target site two. To select for the deletion of 16 nucleotides, AS-primer MT-1.2 is used and for the corresponding wildtype allele primer WT-1.2. For all PCR reactions a common reverse primer is used (R-1) (C) Genotyping results using AS-primers for the deletion at target site two (MT-2) and the corresponding wildtype

allele (WT-2). R-2 is used as reverse primer. (D) Expected genotypes due to PCR results. Boxes with dotted lines symbolized the deletion site, boxes with solid lines the deletion.

Conclusion

Our paper describes a simple and efficient workflow for the generation of clonal, CRISPR/Cas9-edited *Drosophila* cell lines. The technique could essentially be used as is for most Drosophila cell lines; the parts which may need to be adapted are the method of transfection and the way in which single cell clones are isolated. Combining the speed and scale of sequenced cell pools with the effectiveness of AS-primers allows researchers to identify and select clones with a suitable mutation profile in little time. We have demonstrated the general applicability of our approach by generating 8 clonal cell lines mutant for *arm*.

Material and Methods

Cell culture and transfection

Drosophila Kc167 cells (DGRC) were grown at 25°C in M3 + BYPE medium supplemented with 5% fetal bovine serum (FBS) (Gibco) containing 1% penicillin, streptomycin (Sigma). For the transfection, $2x10^6$ cells per well in 2 ml medium in 6 well plates were seeded and transfected with a gRNA-Cas9 expression vector (pAc-sgRNA-Cas9 expression vector from Ji-Long Liu Addgene #49330) using Fugene HD (Promega) transfection reagent according to manufacturer's protocol. We used a 1:2 ratio reagent to vector with a total of 2 µg vector for each well. We recommend including transfection controls (e.g. a GFP plasmid) to monitor the transfection efficiency. Cells were selected in medium containing 5µg/ml puromycin (P8833 Sigma) for 5 days. After selection, cells were washed two times with Phosphate-buffered saline and cultivated in medium without selection marker. The region of the gene to target was determined using the tool http://www.flyrnai.org/crispr2/ and oligonucleotides were designed and cloned as described in manufacturer`s protocol.

Detection of NHEJ events by PCR and Sanger sequencing

After selection on puromycin an aliquot of approximately 1000 transfected cells were assayed for genomic modifications at the cleavage sites within the *arm* locus (FBgn0000117) (Fig 2). Genomic DNA was extracted (e.g. by using DNA-purification kit from Macherey and Nagel) and subjected for a 50 µl PCR reaction using primers spanning over the cleavage sites (Table S1) and the GoTaq2-DNA polymerase (Promega). The GoTaq2-polymerase generates sticky ended 3' A-tailed fragments, so that PCR amplicons could be subsequently cloned into the pGEM®T Easy Vector

System (Promega), which has compatible 3´-T overhangs at the insertion site. Next, we examined 10 colonies obtained by positive blue-white selection with Sanger sequencing and analyzed the sequencing results using the sequence viewer CLC Workbench.

Cloning by limiting dilution in conditioned medium

Clonal *Drosophila* cell lines were obtained according to the Linquist protocol which combines limiting dilution with the use of conditioned medium mixed with fresh medium supplemented with 10% FBS.[34] We harvested conditioned medium from confluent wildtype Kc167 cells grown over 2 days (> 10^6 cells/ml) by harvesting the cells using centrifugation at 3000 g, 5 min at room temperature. We recommend not using a cell population grown over night. We carefully removed the supernatant – the conditioned medium – and mixed it with fresh medium (1:1 ratio) and supplemented the mixture with 10 % FBS, 1% P/S. To isolate single cells limiting dilution was performed with an amount of 50 µl per well of a 96 well plate. Lids were closed with parafilm to avoid desiccation. Later that day or the next day wells were identified containing single cells. Cell clones should be identifiable after around 20 days. Once clones have covered half of the well, they were transferred to larger volumes and plates using filtered tips and used for genotyping. Due to the nature of *Drosophila* cells, we recommend to be careful by transferring cell clones to the next bigger plate and rather to wait until they cover more than half of the well.

Design of allele-specific primers for genotyping

Allele-specific primers were designed according to the concept of Liu and colleagues (see main text).[38] General rules for the PCR primer design were applied. Furthermore, we evaluated designed primers using the software AmplifX 1.5.4. by testing for the formation of hairpin loops, dimers and duplex formation.

Genotyping analysis using allele specific primers

Genomic DNA was extract from isolated and expanded cell clones using e.g. DNA extraction kit (Macherey and Nagel). We examined only a small amount of approximately 100 cells due to their sensibility to density. We analyzed the genotype of the clones using a standard PCR reaction with designed AS-primers as well as corresponding reverse primers (STable1). PCRs were performed in a total volume of 20 µl. As negative controls wildtype cells and water were used.

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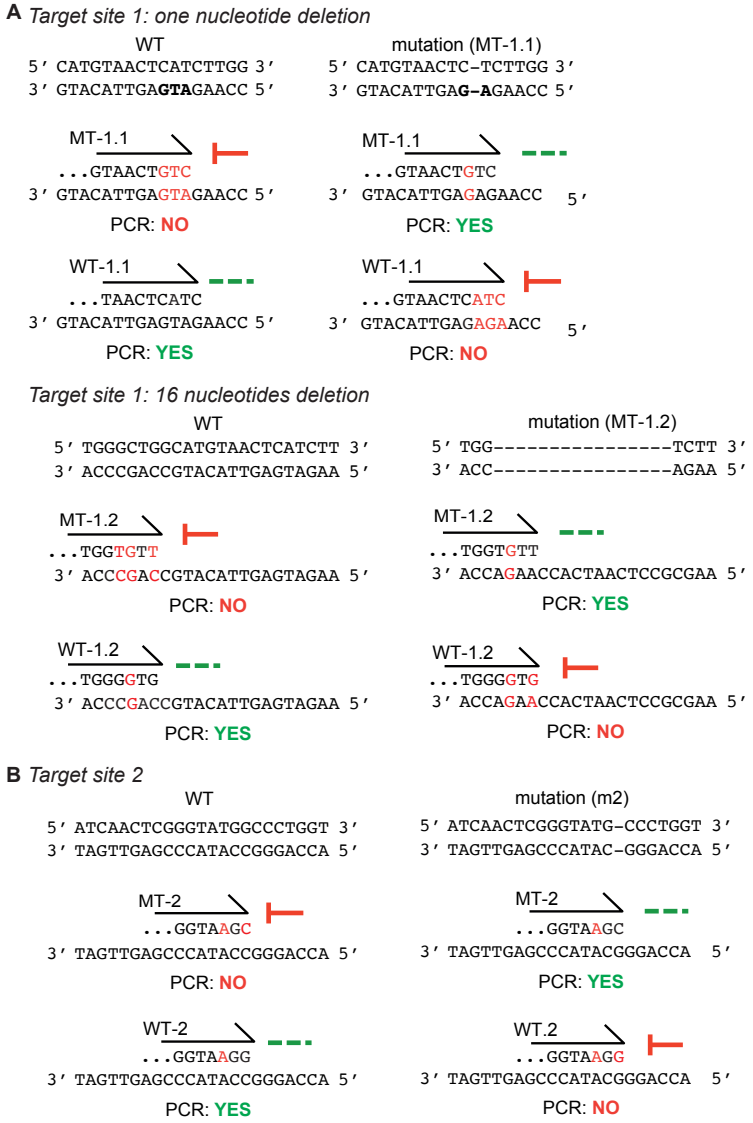
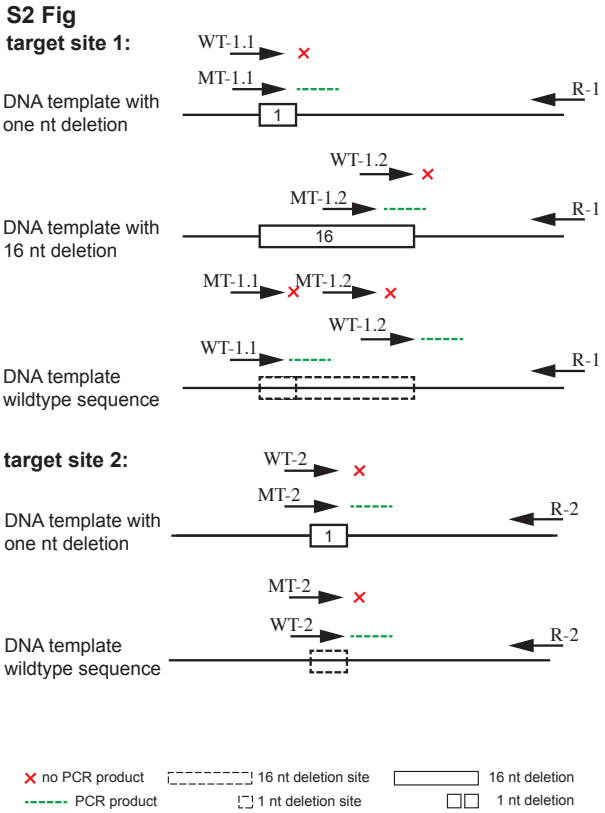
Supplementary figures and figure legends for "Generation of genome-modified *Drosophila* cell lines using SwAP"

Figure S1



S1Figure: Schematic representation of the AS-primer design. (A) Schematic representation of the wildtype and mutant nucleotide sequence harbouring a deletion of one or 16 nucleotides at target site 1. Forward primer MT-1 together with a reverse primer (R-1) (see STable1) will give rise to a PCR product of the mutant allele of one nucleotide. Primer MT-1 harbours three mismatches compared to the wildtype allele and hence cannot amplify the wildtype sequence. Inversely, primer WT-1 matches perfectly with the wildtype allele but harbours three mismatches compared to the mutant allele resulting in no PCR product from the mutant sequence harbouring the deletion of one nucleotide. According to the same principles, AS-primers for the 16 nucleotides deletion (MT-2) and the corresponding wildtype allele (WT-2) were designed. (B) Schematic representation for the AS-primer design for the mutation at target site two (primer MT-3 and primer WT-3) (see STable1 for sequences).



S2Figure: Schematic representation of the binding sites of AS-primers. Red crosses symbolize that respective AS-primer cannot bind; green dotted lines represent the ability to bind at the respective sequence

3.1.2 Studying the role of Toll-7 in Wnt/Wg signaling *in vitro* and *in vivo*

This section presents experiments performed to understand if and how Toll-7 functions in Wnt/Wg signaling *in vitro* and *in vivo*.

Introduction

In our recent work described in chapter 3.1 (Franz et al., 2017), we reported the identification of *Toll-7* as Wnt/Wg target gene in *Drosophila* Kc167 cells. Interestingly, *Toll-7* exhibited the highest fold change of expression after the administration of Wingless (Wg) ligand (WCM vs. CM) and in Wg-signaling deficient *pangolin* null mutant cells, the *Toll-7* gene was de-repressed. These features are reminiscent of the well-known Wnt/Wg target gene *naked cuticle* (*nkd*), an inducible negative feedback regulator of the Wnt/Wg pathway (Zeng et al., 2000; Rousset et al., 2001). *Nkd* was the highest up-regulated in the Wnt ON and most de-repressed gene in the wnt OFF state when Pan was absent. These findings are also intriguing as they suggest that Toll-7 could have a role in the Wnt/Wg signaling pathway. Indeed, this assumption was strengthened by studies showing that Toll-7 is involved in wing development: Overexpression experiments of Toll-7 under the *dpp-Gal4* driver resulted in abnormal wing disc folding as well as bifurcation in adult wings (Yagi et al., 2010). Using *in-situ* hybridization, the authors showed in addition the expression pattern of Toll-7: it is expressed around the wing pouch and the hinge region of the *Drosophila* wing disc Yagi et al. (2010). Moreover, in the *Drosophila* embryo, like engrailed, the striped expression of Toll-7 is posterior to the wg stripes (Kambris et al., 2002). Therefore, in three systems *Toll-7* expression is controlled by Wnt/Wg signaling .

Besides its function for wing development and the developing CNS (McIlroy et al., 2013; Ward et al., 2015), little is known about the roles of Toll-7. Toll-7 belongs to the *Drosophila* Toll receptor family which comprises nine proteins (Tauszig et al., 2000; Imler and Hoffmann, 2001; Valanne et al., 2011), with Toll as the founder of the protein family. Toll is best known for its critical functions in the innate immune response of *Drosophila* to fungal and gram-positive bacterial infections (Lemaitre et al., 1996). This function that was later shown to be conserved throughout the animal kingdom, e.g. it is also executed by the Toll-like receptor superfamily in mammals (Medzhitov et al., 1997; Takeda and Akira, 2005). However, if Toll-7, and the other family members, also have a role

in in innate immunity has remained unclear. Indeed the involvement of Toll-7 in this process is controversial. While two studies suggested that the receptor is involved in virus recognition and restricts virus infection by triggering antiviral autophagy (Moy et al., 2014; Nakamoto et al., 2012), a more recent report could not reproduce these observations (Lamiable et al., 2016). Consistent with Toll-7 not playing an essential role in innate immunity, the earlier study by Yagi and colleagues showed, that the viability of *Toll-7* mutant flies and the induction of antimicrobial peptides after septic injury is comparable to wild-type flies (Yagi et al., 2010).

Hence, we decided to carry out gain-of-function and loss-of-function experiments *in vitro* and *in vivo* to better understand the role of Toll-7 in wing development and Wnt/Wg signaling.

Results and Discussion

We first studied the function of Toll-7 in Wnt/Wg signaling in *Drosophila* S2 cells. *Drosophila* S2 cells are an accepted and reliable model system to investigate the role of Toll and Toll-related genes (Tauszig et al., 2000; Chen et al., 2006; Tanji et al., 2007; Nakamoto et al., 2012). To assay if Toll-7 affects Wnt/Wg signaling related gene expression, we used the *wingful* luciferase system, an artificially built reporter, which enables *Drosophila* researchers to study Wnt/Wg signaling activity in *Drosophila* cells (Städeli and Basler, 2005). Interestingly, overexpression of Toll-7 did not significantly influence reporter activity compared to GFP overexpression control (p-value = 0.0989), after activating the pathway with CHIR (see Figure 1; Franz et al., 2017). We used CHIR due to technical reasons, as S2 cells respond to Wg-ligand only when transfected with the cognate receptor. These results suggest that Toll-7 has neither a positive nor negative effect on Wg-induced gene expression in S2 cells.

We also performed gain-of-function and loss-of-function experiments *in vivo*. To investigate if Toll-7 affects Wnt/Wg signaling *in vivo*, we asked whether Toll-7 affects the expression of *senseless* (*sens*). Sens expression is positively regulated by Wnt/Wg signaling and it is expressed in two stripes flanking the dorsal-ventral boundary in the wing discs (Swarup and Verheyen, 2012). In overexpression experiments, we examined late third instar wing discs overexpressing Toll-7 using an *engrailed (en)-Gal4* driver, which confines the expression to the

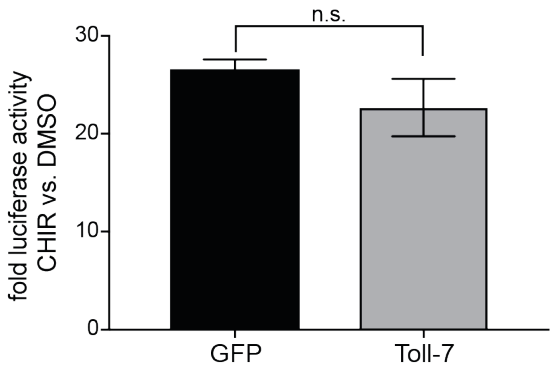


Figure 1: Toll-7 overexpression leads to an increased luciferase activity. Fold luciferase activity of the *wingful* luciferase reporter for control treatment (DMSO) and CHIR after overexpression of Toll-7 and GFP in *Drosophila* S2 cells. See Material and Methods for experimental procedure. Unpaired-T-test was performed using Prism, significance: p-value ≤ 0.05.

posterior compartment of the disc. We found that Sens expression is not affected by Toll-7 overexpression (Figure 2A, see control discs Figure 2B-C). However, we observed that the posterior compartment, expressing higher levels of Toll-7, appeared smaller than the control (anterior) compartment and the posterior compartment in control discs (see Figure 2), indicating that overexpression of Toll-7 may negatively influence the growth of the disc. Interestingly, all discs examined showed extra folds as well as a complete gap between the anterior and the posterior compartment where the cells were detached from one another (see Figure 2A). In addition, Toll-7 overexpression under the *en-Gal4* driver caused small dents or notches within the wing margin of adult wings (see Figure 3). Also Yagi and colleagues have reported an inappropriate folding of the wing imaginal discs when Toll-7 was overexpressed under *dpp* control (Yagi et al., 2010). However, whether any of the observed phenotypes are related to Wnt/Wg signaling remains to be determined.

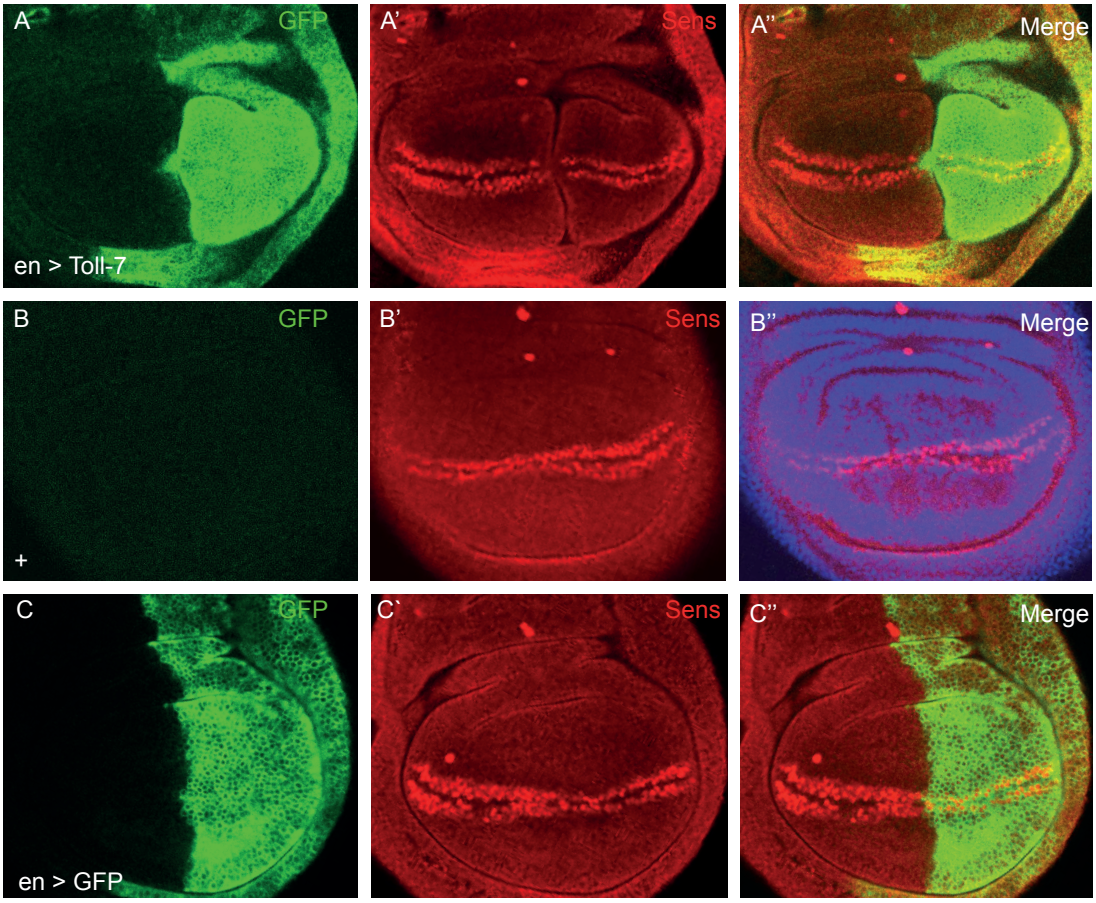


Figure 2: Toll-7 gain-of-function wing discs phenotypes. (A) Wing discs of *en-GAL4:UAS-Toll-7* flies, wild-type (B) *en-GAL4:UAS-GFP* flies (C) stained with anti-Sens (red) antibodies. GFP expression marks the posterior compartment. All discs were analyzed from late third instar larvae. Sens is expressed in two stripes flanking the dorsal-ventral boundary. Wing discs are shown anterior oriented to the left and dorsal to the top.

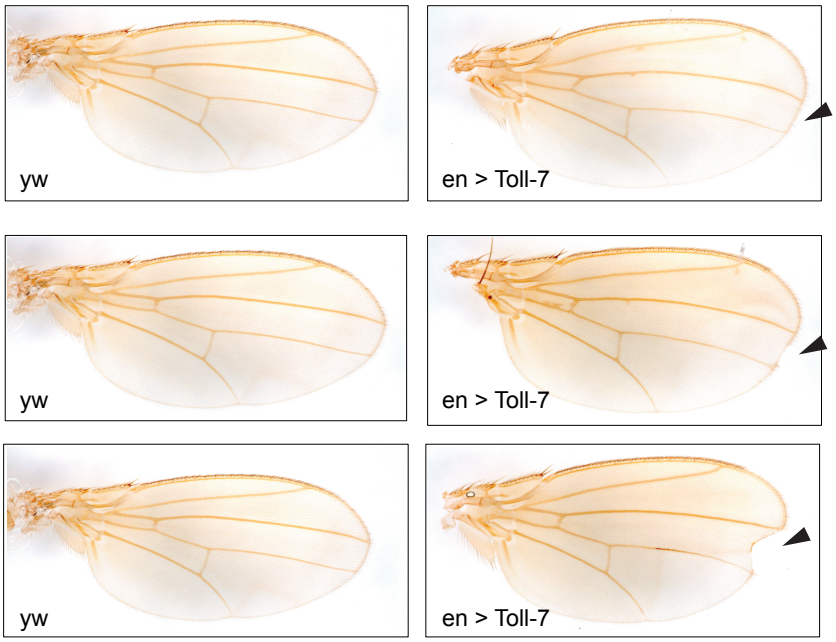


Figure 3: Toll-7 gain-of-function wing phenotypes. Adult wings of *en-GAL4:UAS-Toll-7* flies showing notched in the wing margin with different degree (arrows). As control *yw* flies were used.

We also carried out loss-of-function experiments *in vivo*. We abolished Toll-7 function by generating Toll-7 RNAi clones using the FLP-out Gal4/Gal80 system (see Material and Methods). Clones were marked by the expression of green fluorescent protein (GFP) (Lee and Luo, 2001). We tested three Toll-7 RNAi lines that were available from VDRC (see Material and Methods). When we stained the mutant wing discs for Sens expression, we observed that Sens expression is not affected in clones with reduced Toll-7 expression (see Figure 4). Yet it needs to be noted that the RNAi knockdown is not complete and hence residual Toll-7 expression likely remains in these clones. Therefore, to exclude the possibility that fly stocks carrying constructs designed to knock down Toll-7 did not work properly, loss-of-function studies should be repeated with *Toll-7* null mutant flies. Furthermore, it would be interesting to examine adult loss-of-function phenotypes.

The expression of Toll-7 in the wing disc is strong in the periphery of the wing pouch, but weak or even absent in the center of the pouch (Yagi et al., 2010). Thus, it is not clear whether clones in the center of the pouch would have an effect on Sens expression (in case Toll-7 is not expressed there). Therefore, more Toll-7 loss-of-function clones at the border of the pouch need to be examined,

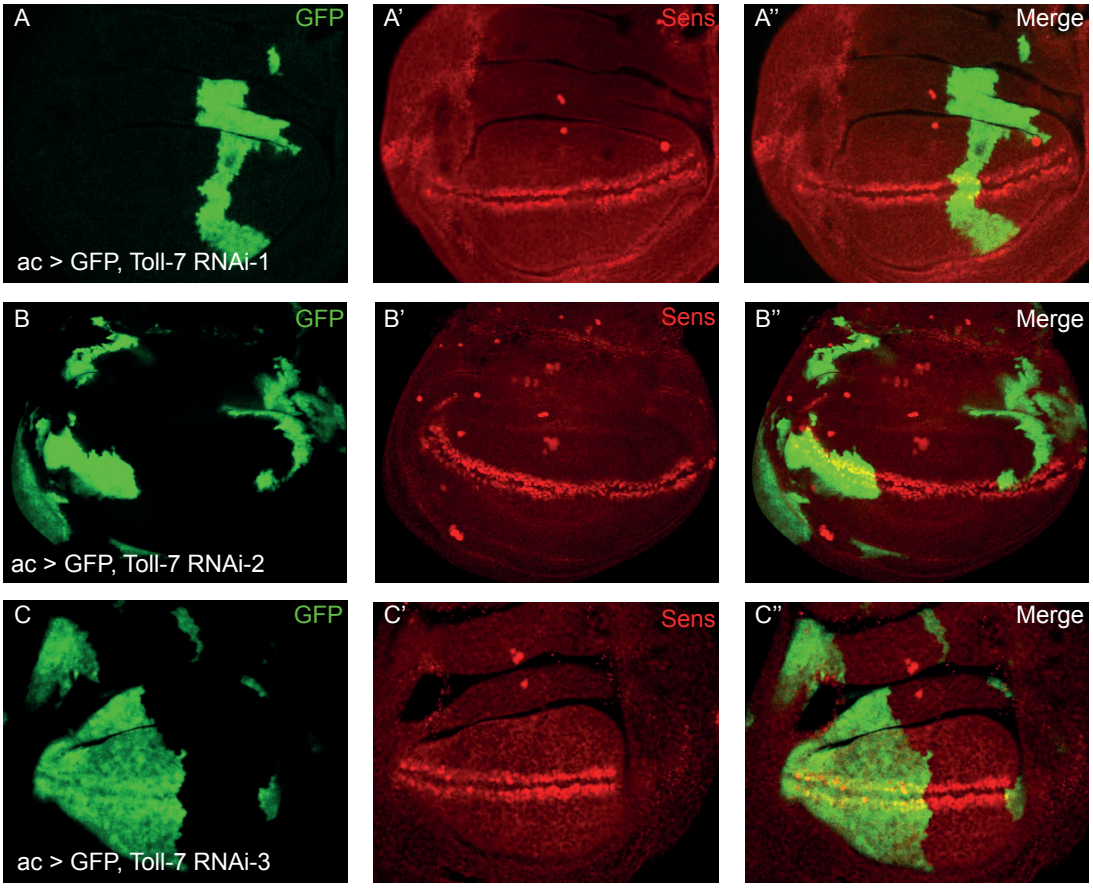


Figure 4: No apparent Toll-7 loss-of-function wing discs phenotypes. (A, B, C) Wing disc carrying Toll-7 RNAi (3 different RNAi lines) clones marked by the presence of green *actin*-GFP expression and stained with anti-Sens (red). In all Toll-7RNAi clones of all three lines, Sens is expressed in two stripes flanking the dorsal-ventral boundary as in wild-type flies.

with a focus on the effects on the Wnt/Wg targets that are activated there, such as *vestigial* (Tabata and Takei, 2004).

Concluding remarks

Our preliminary results indicate that Toll-7 has a role in wing disc growth. This is in agreement with previous studies (Yagi et al., 2010; Kambris et al., 2002). The study from Yagi and colleagues, 2010 demonstrated that Toll-7, Toll-8 and 18W have redundant functions (Yagi et al., 2010). Hence, repeating our experiments with all three candidates may help us to understand in greater detail how Toll-7 operates and if it has any role in Wnt/Wg signaling. Furthermore, there are many questions to be addressed such as, when is *Toll-7* transcription initiated in embryos and how is *Toll-7* expression influenced when

the Wg signaling is switched ON or OFF in the wing disc? Does Toll-7 function via other signaling pathways that regulate wing patterning and growth, such as Dpp or Hedgehog signaling (Baena-Lopez et al., 2012; Kakugawa et al., 2015)? Studying these and other questions in greater detail in the future will shed light on the mechanisms by which Toll-7 orchestrates wing disc development.

Material and Methods

Cell culture and Luciferase assays

Drosophila S2 cells (Drosophila Genomics Resource Center, DGRC) were cultivated at 25°C in M3+BYPE medium, supplemented with 5% FBS (Gibco) and 1% P/S (Sigma). 100.000 cells per well were seeded in a 96 well. A total of 300 ng DNA was transfected per well (100 ng UAS-Toll-7 with 50 ng *actin*-Gal4 and 135 ng *wf*-luciferase with 15 ng *tubulin*-Renilla) in a 1:2 ratio with the transfection reagent Fugene HD (Promega) We transfected the cells according to manufactures protocol. 24 hr after transfection, we activated the Wg pathway for 24 hr with CHIR99021 as described in Franz et al., 2017. Luciferase activities were determined after 48 hr after transfection using the Dual-Luciferase Assay System (Promega).

Drosophila stocks and genetics

The following crosses were carried out: *y w; en-Gal4, Gal80^{ts}* virgins were crossed with males carrying *UAS-Toll-7* (flyORF). For the loss-of-function experiments, we crossed *y w hsp-Flp; Act>CD2>Gal4, UAS-GFP* X *UAS-Toll-7* RNAi (VDRC). All crosses were performed at 25°C except those to generate discs shown in Figure 4, in which larvae were reared at 18°C, the Gal80^{ts} permissive temperature, and then shifted to 29°C, the restrictive temperature, 2 days before dissection to induce UAS-Toll-7 expression. To generate knock-down clones, larvae were heat-shocked for 8 min at 37°C 24 hr AED.

Immunostaining and microscopy

Imaginal discs from third instar larvae were fixed and stained by standard techniques with guinea-pig anti-Senseless (1:800, a gift by the laboratory of Richard Mann, Columbia University), mouse anti-GFP (1:400) as primary antibody and Alexa 488, Alexa 555 and Alexa 647 (1:400, Molecular Probes) were used as secondary antibodies.

3.2 Deciphering the code of Wnt/Wg-responsive enhancers

This section presents a computational analysis of the Wnt/Wg-responsive enhancers reported in Franz et al., 2017. The aim is to understand in greater detail how the regulatory elements operate.

Introduction

Cellular events regulating animal development and tissue homeostasis are controlled by genetic programs - the sets of genes whose orchestrated and combinatorial expression is tightly governed via signaling pathway-specific transcription factors. The Wnt/Wg pathway-specific transcription factor family TCF/LEF provides target specificity by recognizing a short, specific DNA consensus sequence (motif) through its conserved high mobility group (HMG) DNA-binding domain. The genomic regions directly bound by the TCF/LEF-dependent transcriptional complex are defined as Wnt responsive elements (WREs) (Chang et al., 2008). WREs are not only found in the promoter of target genes, but are often located in more distant regulatory regions called enhancers.

Enhancers are *cis*-acting regulatory DNA segments that are typically a few hundred bp in length and can be occupied by several transcription factors in a combinatorial fashion (Spitz and Furlong, 2012). More than 35 years have passed since their discovery. In 1981, the first enhancer was described as a 72 bp DNA element of the viral SV40 genome, which could activate the transcription of the *beta-globulin* reporter gene in HeLa cells (Banerji et al., 1981). Further studies showed then two years later the existence of endogenous enhancers in myeloma cells (Gillies et al., 1983; Banerji et al., 1983). Today, it is well established that enhancers are crucial for precise spatiotemporal gene regulation in developmental systems by providing an operational platform for transcription factors (Blackwood, 1998; Levine, 2010).

In their initial study, Banerji and colleagues observed that the isolated viral DNA fragment functions independently of its distance and orientation in respect to the reporter gene (Banerji et al., 1981) - features that are today considered as the hallmarks of enhancers (Shlyueva et al., 2014a). Further work

has uncovered many other characteristics that are associated with the *cis*-acting DNA regulatory elements, such as: the presence of transcription factor-specific binding sites within the same enhancer (Blackwood, 1998; Lemon and Tjian, 2000) or even clusters of multiple transcription factor binding sites - so called super-enhancers - (Whyte et al., 2013; Lovén et al., 2013), and specific histone variants that contribute to nucleosome plasticity and thus enhancer activity (Bernstein et al., 2005; Heintzman et al., 2007, 2009).

Various methods have been developed to identify new enhancers and their location, leading to the successful prediction of several hundred thousands of enhancers in *Drosophila*, mouse and human cells (Thurman et al., 2012; Consortium et al., 2012). Exploiting the distinctive properties of enhancers, the technologies range from examining genomic sequences for transcription factor binding sites by computational or experimental methods (Allende et al., 2006; Hallikas et al., 2006; Blanchette et al., 2006; Pennacchio et al., 2006; Visel et al., 2008; Farnham, 2009), mapping the genome-wide pattern of DNase I hypersensitive (Gross and Garrard, 1988; Boyle et al., 2008), to techniques such as Chromatin Immunoprecipitation assay followed by deep sequencing (ChiP-seq) (Dorschner et al., 2004). Another method is the chromosome conformation capture (3C) technology (Dekker, 2002) and its derivatives, such as 4C, 5C, Hi-C, ChiA-PET (Fullwood et al., 2009; van Steensel and Dekker, 2010; Jin et al., 2013), wherein one detects the frequency of direct physical interactions between an enhancer and the promoter of the target gene. Other technologies evaluate traditional reporter constructs in large-scale high-throughput approaches. These technologies include enhancer-FACS-sequencing (eFS) (Gisselbrecht et al., 2013), massively parallel reporter assays (MPRA) (Melnikov et al., 2012) and STARR-sequencing (STARR-seq) (Arnold et al., 2013). eFC and MPRA rely on the activity of a reporter gene or the count of fused tag sequences. In contrast, STARR-seq quantifies the activity of each enhancer identified in candidate genomic libraries, by directly measuring the amount of transcripts produced by using RNA-seq. Briefly, the genomic libraries used in this assay consist of a series of genomic DNA fragments covering the entire genome and are inserted between an ORF and a polyA tail. These plasmid clones (each carrying a specific genomic fragment) are then transfected into target cells, and the activity of each enhancer is defined based on the amount of transcript that originates

from that single plasmid (Arnold et al., 2013).

In our previous study, we used the quantitative high-throughput STARR-seq technology to analyze the Wnt/Wg-responsive enhancer landscape on a genome-wide scale in *Drosophila* Kc167 cells. For pathway activation, we applied the small molecule GSK3 inhibitor CHIR99021 (see chapter 3.3, Franz et al., 2017). Following up on our previous study, we now set out to i) understand whether the identified Wnt/Wg-responsive enhancers regulate the expression of Wnt/Wg target genes and ii) analyze the sequence constraints in the TCF/Pan motif to determine the rules that define a Wnt/Wg-responsive DNA regulatory element.

Results and Discussion

The challenge of connecting Wnt/Wg target genes to Wnt/Wg-responsive enhancers

The distance of an enhancer to adjacent promoters, combined with gene expression data, has been used in *Drosophila* to assign enhancers to their putative target gene (Kvon et al., 2014; Shlyueva et al., 2014b). We used this strategy to map Wnt/Wg-responsive enhancers identified in the STARR-seq experiments to Wnt/Wg target genes. A comprehensive list of 51 Wnt/Wg target genes was generated in our recent study when using large-scale RNA-seq approaches with the Wingless-ligand as stimulus for Wnt/Wg pathway activation (see chapter 3.1, Franz et al., 2017). Using the method described by Kvon and colleagues, enhancers were mapped to the Wnt/Wg target genes within their 20 kb radius (Kvon et al., 2014). 12 of the 344 identified enhancers could be matched to a promoter of a Wnt/Wg target gene, which are 23.5% of Wnt/Wg target genes (see Figure 1A). Interestingly, all enhancer-gene pairs correlated positively (Pearson correlation $R^2 = 0.7775252$, p-value = 0.002914): up-regulated genes clustered with induced enhancers, whereas down-regulated genes with repressed ones (see Figure 1A). Moreover, strongly induced enhancer (log2ratio > 3) mapped close to highly up-regulated genes, such as *nkd* and *Toll-7* (log2ratio $>$ 4; see Figure 1A). The analysis described above integrated data sets derived from different activating stimuli: CHIR for STARR-seq and WCM for RNA-seq. We also compared identified enhancers with the expression profile of genes activated only with the synthetic GSK3 inhibitor CHIR (see chapter 3.3) to understand

in greater detail how the enhancers regulate gene expression. We compared significantly CHIR-deregulated genes (p-value ≤ 0.0005 and fold change of 2) with enhancers identified via STARR-seq. Consistent with the previous analysis we found that 25% of the significantly up-regulated genes could be associated with an induced enhancer, whereas 7% were associated with a repressed DNA regulatory element. *Vice versa*, 27% of down-regulated genes were connected to repressed enhancer and 6% to an induced one (see Figure 1B).

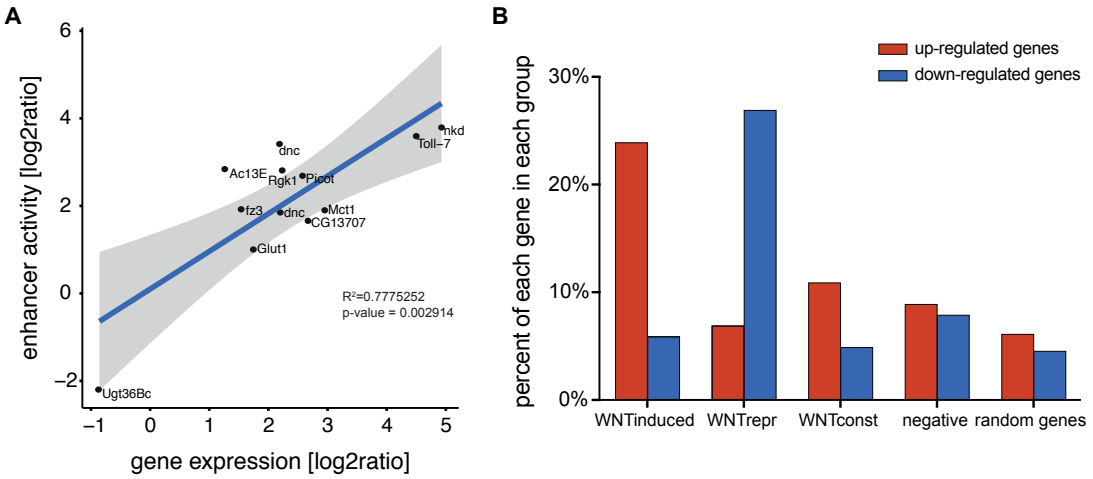


Figure 1: Enhancer-to-gene assignment. (A) Scatterplot of enhancer activity (y-axis) versus Wnt/Wg target gene expression (WCM vs. CM; x-axis). A linear regression line is marked in blue including the 95% confidence region in grey. Correlation was calculated with using Pearson correlation. (B) The percent of assigned genes from the CHIR-data set that are significantly altered (p-value ≤ 0.0005) and at least 2-fold up- or down-regulated for each class of enhancers are schematically depicted.

In the above analysis the maximum distance between an enhancer and target gene is arbitrarily set at 20 kb. However, enhancers can act over a very long range, for review see (Whitaker et al., 2015). Additionally, a recent study identified so-called shadow enhancers that have redundant functions in *Drosophila* mesoderm development (Cannavò et al., 2016). Therefore gene regulation mediated by enhancers has a complex combinatorial nature. More work is needed to determine precise enhancer-to-gene linkages, e.g. by mutating the endogenous enhancer locus using CRISPR/Cas9 and monitoring its effect on gene expression, or by altering the activity of an enhancer epigenetically (Mendenhall et al., 2013; Kearns et al., 2015). Such approaches have been used successfully to validate the mouse promoter-enhancer interactions by

combining ChIA-PET with TALEN-mediated genome editing (Kieffer-Kwon et al., 2013). Another study characterized the genome-wide action of enhancers in a mammalian cell culture system by using STARR-seq in combination with CRISPR/Cas9 (Dao et al., 2017).

A caveat of the STARR-seq method is that it is based on a reporter assay technology in which the epigenomic context is possibly not representative of the native structure of the chromatin (Arnold et al., 2014). Therefore other levels of gene regulation, such as small and long non-coding RNA molecules (Holoch and Moazed, 2015), insulators and silencers (Levine and Tijan, 2003) may not influence gene expression.

Strongly induced Wnt/Wg-responsive enhancers possess defined combinations of TCF/Pan motif and Helper site variants

Analyzing the motifs present within regulatory elements is a common approach to begin to decipher the requirements for activation. Transcription factor-specific motifs are usually 4 - 11 bp in length, and summarize DNA binding preferences of transcription factors. The most frequent base at each position are given however base substitutions may be tolerated at so-called degenerate positions (Stormo and Zhao, 2010). A recent study analyzed such degenerate motif patterns in the *Ciona*-Otx-a enhancer and demonstrated that "suboptimal" motifs (motifs that lead to a lower affinity between DNA and the transcription factor) are in fact sufficient to drive tissue-specific gene expression, whereas optimal sites lead to stronger ectopic expression (Farley et al., 2015). To explore the variability and constraints of the TCF/Pan site motif (normally indicated as CCTTTGATCTT, (van de Wetering et al., 1997) we carried out detailed computational motif analysis on the enhancers found in the STARR-seq experiments (see Material and Methods).

Our recent study showed that the TCF/Pan consensus sequence is significantly enriched in induced enhancers (2.7-fold enrichment, p-value = $1.3x10^{-8}$) but is not present in repressed elements (see chapter 3.1, Franz et al., 2017). To test for the presence of a more degenerate consensus sequences, we computationally constructed 36 possible TCF/Pan motif versions that are 4 - 11 bp in length (see Figure 2A), and determined whether these motif variations were significantly

enriched within induced enhancers. We found 17 TCF/Pan motif variants were significantly enriched (p-value ≤ 0.005) in comparison to control sequences (see Figure 2B). Interestingly, our analysis revealed that highly active enhancers, when compared with more mildly induced ones, were mainly associated with longer motifs consisting of 7 to 10 nucleotides (see Figure 2C), highlighting the importance of the TCF/Pan-DNA affinity for a stronger expression.

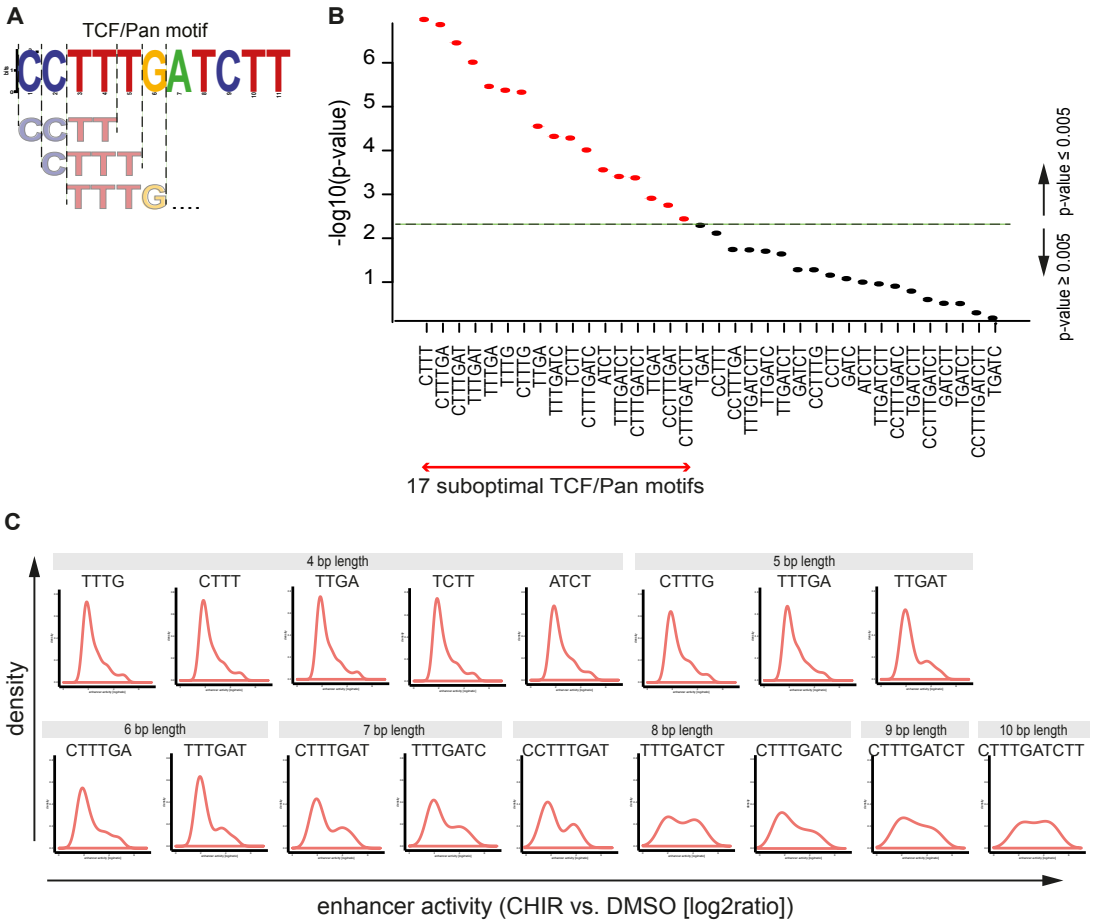


Figure 2: Induced Wnt/Wg-responsive enhancer have suboptimal TCF/Pan binding sites. (A) Schematically representation of suboptimal TCF/Pan motifs generation. (B) Scatterplot showing the statistical significance (y-axis; -log10 p-value) of identified suboptimal TCF/Pan motifs. 17 motif versions were significantly enriched (p-value ≤ 0.05) (red dots) in induced enhancer sequences in comparison to control sequences (see Material and Methods). Black dots represent not significantly enriched motifs. Significance was computed using Fisher exact test (see Material and Methods). (C) Density plots depicting the distribution of enhancers over their activity (x-axis) for each motif. Peaks display where values are concentrated over the interval.

In our recent study, we found the Helper site to be significantly enriched in induced Wnt/Wg-responsive enhancers (see chapter 3.1, Franz et al., 2017).

The Helper site is a GC-rich element found in close proximity to the TCF/Pan binding motif and important for optimal Wnt/Wg target gene activation (Chang et al., 2008). Using the same approach employed for the TCF/Pan motif analysis, we constructed a library of Helper site variants. We found several of them were significantly enriched in our data set (p-value ≤ 0.005; data not shown). Next, we asked which motifs variants of TCF/Pan and Helper site co-occur within a single enhancer and whether these co-occurring motifs affect enhancer strength. We found 9 out of 17 TCF/Pan motif versions co-occurred with a variant of the Helper site (see Figure 3). Additionally, we found that with increasing lengths of both motifs, TCF/Pan and Helper, the activity of enhancers increased (see Figure 3) and that surprisingly, some strong enhancers had predominantly either long TCF/Pan motifs in combination with short Helper site variants or short TCF/Pan sites with long Helper site version (see Figure 3). This latter finding is unexpected and suggests a more prominent role for the Helper site than anticipated. Interestingly, a recent report has already proposed that TCF1/TCF7 can mediate transcriptional regulation via Helper sites alone independent of readily identifiable canonical WRE motifs in DLD-1 colon cancer cells (Hoverter et al., 2014). However, in our motif discovery analysis we included also short motif versions found in long versions, thus an essential next step is to exclude those from the analysis to understand in greater detail how the Helper site contribute to the activity of enhancers.

A critical next step will also be to test the findings experimentally, for example by using mutagenesis assays and/or luciferase reporter systems to directly quantify the activity of the different motif variants we identified. Moreover, additional work concerning the orientation and the spacing between the motif sequences may also reveal mechanistic insights into the motif requirements for Wnt/Wg-responsive enhancers, similar to the findings described in (Archbold et al., 2014). *De novo* motif discovery might also shed light onto the control Wnt/Wg target gene expression, and if this is mediated by a co-operation of Pan with other transcription factors. Indeed, recent studies have shown that Pan as well as vertebrate TCF family co-occupy enhancers with several other transcription factors (Junion et al., 2012; Verzi et al., 2010; Trompouki et al., 2011; Zhao et al., 2016). Hence, integrating these results with ours would be a potential next step.

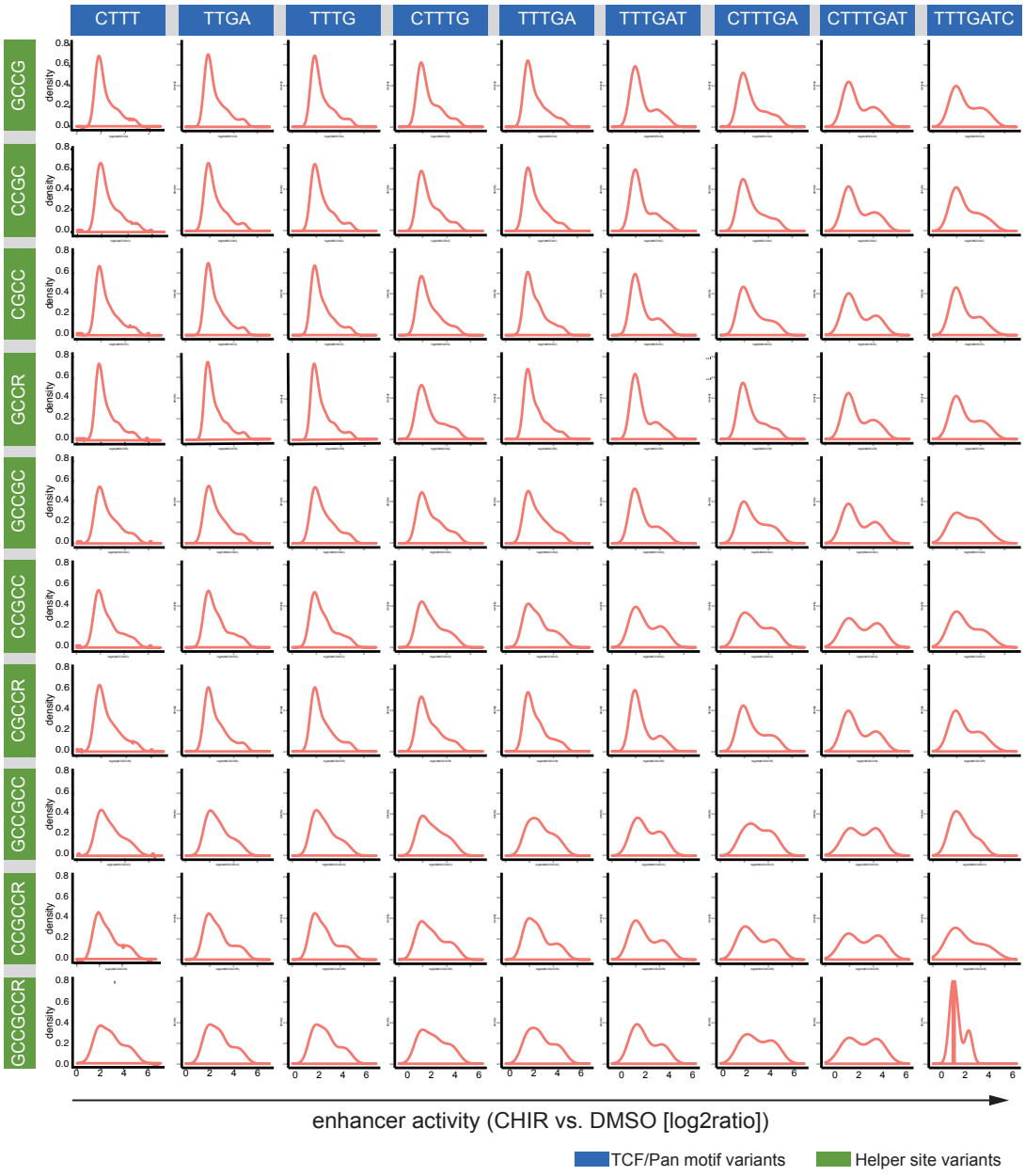


Figure 3: Co-occurrence of TCF/Pan and Helper site motif versions. Density plots showing the distribution of enhancers (y-axis) possessing respective motif pair over their activity (x-axis; log2 ratio CHIR vs. DMSO) for all possible motif combinations. In green Helper site variants, in blue TCF/Pan binding site variants.

Repressed enhancers contain non-conventional TCF/Pan binding sites

In contrast to induced enhancers, repressed enhancers were neither significantly enriched for the classical TCF/Pan motif nor the Helper site (see chapter 3.1, Franz et al., 2017). When analyzing the sequences for the occurrence of constructed TCF/Pan motif variants, we found 5 short (4 bp long) TCF/Pan motif variants were significantly enriched within repressed enhancers, in comparison to control regions (p-value ≤ 0.05) (data not shown, see Material and Methods). Almost all repressed enhancers exhibited these short motifs (see Figure 4A). An alternative TCF/Pan binding site AGAWAW (W=A/T) has been previously reported to mediate the repression of the Wnt/Wg target gene *Ugt36Bc* (Blauwkamp et al., 2008). This prompted us to test whether Wnt/Wg repressed enhancers possess a novel putative regulatory site. We found 3 significantly enriched motifs that could be alternative TCF/Pan binding sites. These motifs were present in almost all repressed enhancers, with the AGATAA sequence in strong repressors (see Figure 4B). Further experimental work is required to test whether TCF/Pan motif variants are essential for the repressive mode of action of enhancers and to address whether Pan or other transcription factors bind to the identified motifs. Our list of repressed Wnt/Wg-responsive enhancers is in addition an ideal starting point to clarify the recently proposed highly degenerate Helper-like motif (KCCSSNWW [K=G/T, S=G/C, N=any base, W=A/T]), which has been found to be paired with the alternative TCF/Pan binding site to mediate the repression of *Tiggrin* in *Drosophila* (Zhang et al., 2014).

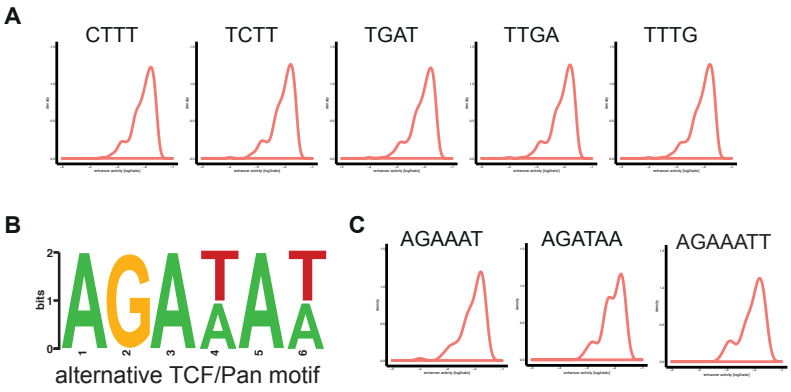


Figure 4: Motifs discovered in repressed enhancer. Density plots showing the distribution of repressed enhancers over their activity (x-axis) for suboptimal TCF/Pan motifs (A) and alternative TCF/Pan binding sites (C). (B) PWM logo for the alternative TCF/Pan motif.

These novel observations and future studies, together with our previous finding that repressed enhancers are strictly dependent on Pan (Franz et al., 2017), constitute the basis for future investigation into the yet poorly understood mechanism of Wnt/Wg-mediated repression (Affolter et al., 2008).

Material and Methods

Motif discovery

To identify the most relevant motifs in each induced and repressed sequence sets, we searched for motif signals in the control region (-1000 ... -500) compared to the test region (-250 ... 250) relative to the sequence loci. We then selected the top motif (Fisher exact test).

Computational analysis

Computational analysis of the motifs was carried out using R as well as all statistical tests. Plots were created using R and Illustrator.

3.3 Activating the Wnt/Wg pathway: a comparison between transcriptional changes triggered by GSK3 inhibitor CHIR99021 and Wingless-conditioned medium

In this section, we investigated to what extent the synthetic small agent GSK3 inhibitor CHIR99021 mimics the Wnt/Wg response in *Drosophila* Kc167 cells using large-scale quantitative RNA-sequencing approaches.

Introduction

The precise regulation of the cytosolic and nuclear levels of beta-catenin - the obligate transcriptional activator of Wnt/Wg target gene expression - by the destruction complex is a central feature of Wnt/Wg signaling. In the Wnt/Wg OFF state, beta-catenin is marked for proteasomal degradation by this multi-protein complex, whereas in the Wnt/Wg ON state beta-catenin levels rise as the destruction complex disassembles, for review see (Macdonald and He, 2016). Key constituents of the destruction complex include Axin, adenomatous polyposis coli (APC), casein kinase 1 alpha (CK1alpha) and glycogen synthase kinase 3 beta (GSK3beta) (Aberle et al., 1997; Ikeda et al., 1998; Hart et al., 1998). They induce the degradation of beta-catenin via phosphorylation: following the phosphorylation at Ser45 by CK1alpha, GSK3beta phosphorylates beta-catenin at Thr41, Ser37 and Ser33 residues (Liu et al., 2002). The tightly control of beta-catenin levels by the destruction complex is thus essential for regulated Wnt/Wg signaling. Unsurprisingly, inappropriate beta-catenin stabilization and target gene expression due to mutations in the destruction complex have been frequently observed in Wnt-related pathologies; including degenerative and neurological diseases as well as carcinomas, for review see (Herr et al., 2012; Kahn, 2014). Mutations in APC provided even the first link of Wnt/Wg signaling with disease (Kinzler et al., 1991; Groden et al., 1991). Other examples are an inhibition of GSK3beta, which has been associated with neurodevelopmental defects (Mao et al., 2009) and beta-catenin-stabilizing mutations identified in various human neoplasms including colon cancers (Morin et al., 1997; Iwao et al., 1998), hepatocellular carcinomas (Miyoshi et al., 1998), human skin tumors (Rubinfeld, 1997; Chan et al., 1999) and prostate cancer (Voeller et al., 1998). Other examples are loss-of-function mutations in Axin which have been found

in hepatocellular carcinomas (Satoh et al., 2000) and colorectal cancer (Lammi et al., 2004).

Components of the destruction complex have and are being exploited as therapeutic agents. Some pathway-inhibiting drugs disrupt the function of Axin, and most pathway-activating agents target GSK3, for review see (Nusse and Clevers, 2017). Among the long list of GSK3 inhibitors, CHIR99021 (CHIR) has been described as the most potent and selective (Bain et al., 2003, 2007). A synthetic purine analog, CHIR is an ATP-competitive inhibitor for GSK3, and effectively inhibits GSK3 in nanomolar concentrations (Ring et al., 2003). A caveat of activating the Wnt/Wg pathway with GSK3 inhibitors is that additional pathways will be triggered, because GSK3 is involved in a wide array of cellular processes (Coghlan et al., 2000). GSK3 is a serine/threonine kinase and was originally identified for its ability to phosphorylate and inhibit glycogen synthase in insulin-mediated glycogen synthesis (Embi et al., 1980). However, further studies showed soon that GSK3 is a versatile kinase occupying a central stage in many cellular events by modulating the activity of key regulatory proteins involved in various signaling pathways, such as insulin signaling (Eldar-Finkelman and Krebs, 1997), Hedgehog signaling (Jia et al., 2002; Price and Kalderon, 2002), translation (Welsh et al., 1998), transcription (Nikolakaki et al., 1993; Fiols et al., 1994; Beals, 1997) and cytoskeletal regulation (Sperber et al., 1995; Hong et al., 1997).

CHIR-induced Wnt/Wg pathway activation was demonstrated in mouse embryonic stem cells and human lung tissues by quantifying the mRNA levels of known Wnt/Wg target genes and using the Topflash assay (Ying et al., 2008; Li et al., 2011; Zhang et al., 2012; Naujok et al., 2014). Topflash is an artificial luciferase reporter widely used for measuring the levels of Wnt/Wg signaling (Molenaar et al., 1996). Therefore, it is still unclear, to what extent CHIR stimulation mimics the Wnt/Wg transcriptional response if one looks on a genome-wide scale.

Hence, to address this, we compared the genome-wide CHIR-triggered transcriptome with the genome-wide profile stimulated by Wingless (Wg) (see chapter 3.1, Franz et al., 2017) in *Drosophila* Kc167 cells .

Results and Discussion

To first identify on a genome-wide scale CHIR-regulated target genes, we performed next-generation RNA-sequencing (RNA-seq) in *Drosophila* Kc167 cells treated with CHIR in three biological replicates. *Drosophila* cells were stimulated for 24 hr with 25 μM of the GSK3 inhibitor. As control we used cells treated with DMSO (see Material and Methods). These conditions are optimal for stimulating the expression of Wnt/Wg pathway targets in *Drosophila* cells (see chapter 3.1, Franz et al., 2017). CHIR treatment led to the induction of 1089 significantly altered genes (p-value $\leq$ 0.0005, FDR-corrected p-value $\leq$ 0.05; see Figure 1A). We further analyzed our large-scale data set with the PANTHER (protein annotation through evolutionary relationship) classification system (Mi et al., 2013) to discover in which biological processes the affected genes are implicated. As expected, the analysis showed that target genes were involved in many diverse biological processes, including metabolic, cellular and developmental activities (see Figure 1B).

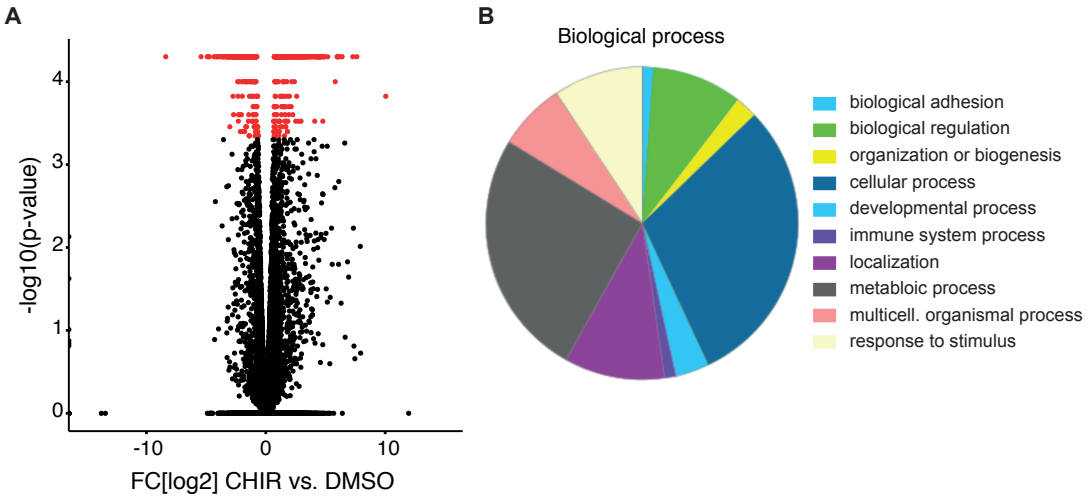


Figure 1: Gene expression profile in *Drosophila* Kc167 cells after CHIR treatment. (A) Volcano plot showing the log2 fold change (x-axis) and statistical significance (y-axis; -log10 p-value) of CHIR-responsive genes. 1089 genes were significantly differently expressed after CHIR treatment (p-value $\leq$ 0.0005; red dots). Black dots represent all genes that showed an altered expression profile. (B) Gene ontology biological process classification of annotated genes in percentage using PANTHER (protein annotation through evolutionary relationship).

Next, in order to understand how reliably CHIR reflects the Wnt/Wg response, we compared the gene expression profile triggered by CHIR with the genome-wide transcriptome activated by Wingless-conditioned medium (WCM)

(van Leeuwen et al., 1994). For this comparison, we used the already described WCM-induced RNA-seq data set from our previous study (see chapter 3.1, Franz et al., 2017), in which we stimulated *Drosophila* Kc167 cells for 24 hr with WCM, as control, conditioned medium without Wg-ligand was used. As for the CHIR data set, we applied a p-value ≤ 0.0005 (FDR-corrected p-value ≤ 0.05) for defining significantly Wg-deregulated genes. We found 85 (data not shown). Surprisingly, by comparing the two data sets (CHIR and WCM), we found only a small overlap of 48 genes (see Figure 2A). Even more unexpected, the correlation between the genes was weak with a coefficient of $R^2 = 0.56$ (p-value = 0.0008) (see Figure 2A), as the fold change of expression of many genes (31%) did not correlate: genes that were up-regulated following CHIR induction were down-regulated after WCM treatment and *vise versa* (see red circles in Figure 2A). However, we found among the genes whose change of expression correlated well-studied Wnt/Wg target genes such as *naked cuticle* (*nkd*), *CG6234*, *frizzled 3* (*fz3*) and *Peroxidasin* (*Pxn*) (Zeng et al., 2000; Sivasankaran et al., 2000; Fang et al., 2006; Blauwkamp et al., 2008). Focusing on these genes, we performed a hierarchical cluster analysis based on the gene expression profiles to investigate in greater detail how the genes are regulated by the two stimuli. We found that this set of genes was very similarly expressed after WCM and CHIR stimulation, however with different activation levels: strongly activated genes by WCM with a fold-change ≥ 3 were among the strongest expressed genes following CHIR treatment exhibiting a fold-change ≥ 4 (see green branches of dendrogram, Figure 2B). The highest Wg-induced genes *nkd*, *Toll-7* and *axo* exhibited an equally strong induction upon CHIR stimulation (violet branches of dendrogram, Figure 2B) and similarly, genes with low Wg-induction of expression profiles were only mildly affected by CHIR (see orange branches of the dendrogram, Figure 2B). In contrast, the vast majority of down-regulated genes showed a weak Wg-mediated repression, which was much stronger following CHIR treatment (see grey branches of dendrogram, Figure 2B).

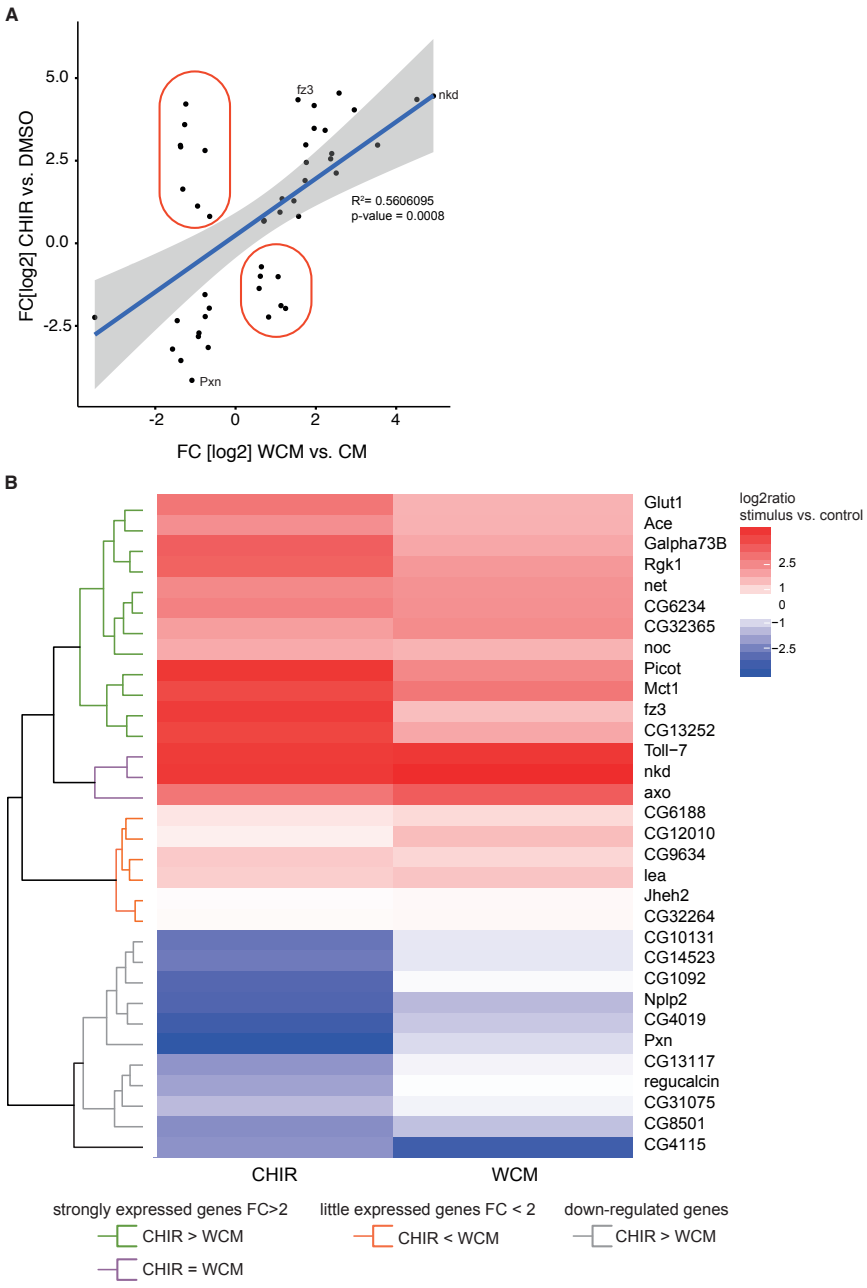


Figure 2: Comparison of WCM and CHIR gene expression data. (A) Scatterplot shows fold induction for CHIR-treated (y-axis) versus WCM-treated (x-axis) genes. A linear regression line is marked in blue including the 95% confidence region in grey. Correlation was calculated with using Pearson correlation. (B) Hierarchical clustering was performed using R on genes found in both, WCM and CHIR RNA-seq data sets with a positively correlating expression profile. Heat map of those genes showing their log2 fold change (FC) of expression after WCM or CHIR treatment versus respective control treatment. Up-regulated genes are shown in red, down-regulated genes in blue.

These findings indicate that in general CHIR activates target genes stronger. However, to exclude the possibility that the observed divergent expression profiles are of technical nature brought about, for example, by the challenge of producing WCM in large quantities, future work should focus on investigating whether target gene expression profiles can be adjusted between WCM and CHIR treatment by modulating inhibitor concentration, WCM production procedures and/or altering the timing of stimulation. Another critical next step could be to investigate the function of identified target genes to understand how they contribute to the biological output of the Wnt pathway. For example to establish if they are potential feedback regulators.

Next, we investigated, how representative the 48 genes whose expression changed in response to WCM and CHIR were for both datasets. The overlap represented about half (55%) of the genes affected by WCM but only 4% of the genes whose expression is changed in response to CHIR (see Figure 3A). The fact that many more genes are changed by CHIR than by WCM suggests that taken as whole CHIR does not only mimic the spectrum of the Wg-triggered Wnt/Wg response in *Drosophila* Kc167 cells. Its effect is much broader.

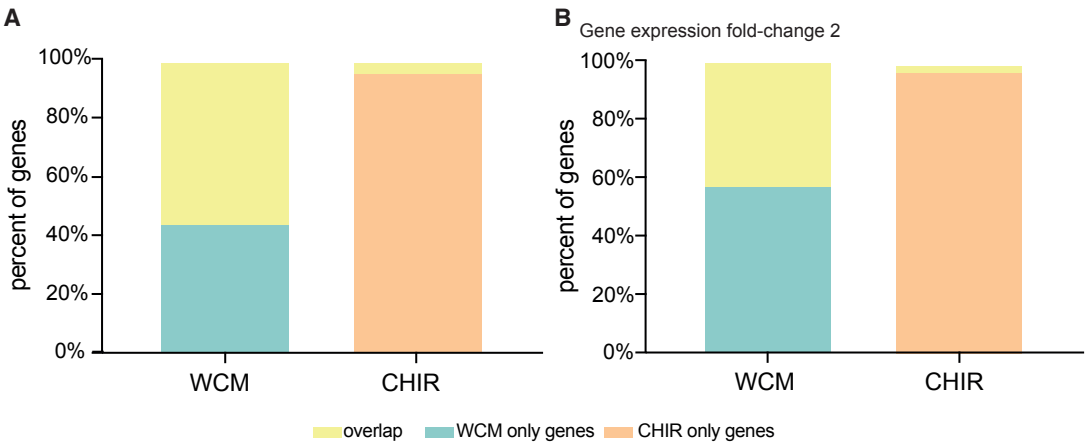


Figure 3: Proportion of common genes within the respective data set in percentage. (A) Proportion of common genes in the WCM and CHIR data set. Yellow represents proportion of overlapping genes (48 genes), green are genes only found in WCM data set (37 genes), orange genes only found in CHIR data set (1041 genes). (B) Proportion of genes exhibiting a fold-change threshold of at least 2. Yellow represents proportion of overlapping genes (22 genes), green are genes only found in WCM data set (29 genes), orange genes only found in CHIR data set (653 genes).

In our recent publication, we defined the Wnt/Wg target genes in *Drosophila* Kc167 cells by setting a fold-change threshold of 2, which led to the identification of 51 Wnt/Wg genes that were significantly differently expressed after stimulating the Wnt/Wg pathway with WCM (see chapter 3.1, Franz et al., 2017). We applied this threshold to the CHIR data set to investigate, how well the previously defined Wnt/Wg signature is repeated by CHIR-induction. The analysis revealed that 674 genes are at least 2-fold up- or down-regulated and there is an overlap of 22 common genes between both data sets (data not shown). This overlap represents 43% of all WCM and 3.2% of the CHIR regulated genes (see Figure 3B), demonstrating clearly that CHIR cannot reliably mimic the Wnt/Wg signature in this system.

Together, these results show that GSK3 inhibition has pleiotropic effects and is only able to partially reproduce the Wnt/Wg response that is triggerd by the Wg-ligand in the WCM. Hence, our data show that using CHIR for large-scale RNA-seq approaches to study the Wnt/Wg pathway needs to be approached with caution and results carefully interpreted. A critical next step is to extend our findings to the mammalian system, as CHIR is widely used as alternative inductor of the Wnt/Wg pathway in this system (Ying et al., 2008; Li et al., 2011; Zhang et al., 2012; Naujok et al., 2014).

Material and Methods

Drosophila cell culture

Drosophila Kc167 cell lines were grown in M3+BYPE medium, supplemented with 5% fetal bovine serum (FBS) and 1% penicillin and streptomycin at 25°C.

Activating Wnt/Wg signaling in *Drosophila* Kc cells

To induce the Wnt/Wg signaling pathway with CHIR99021 (S1263, Selleckchem), 25 μM of the inhibitor was used and added to the medium for 24 hr. As control DMSO was used. After 24 hr of induction, cells were harvested. Activating the Wnt/Wg signaling response with WCM is described in chapter 3.1, Franz et al., 2017. Briefly, WCM was harvested from S2 tubulin wingless cells. S2 tubulin wingless cells were seeded 24 hr prior collecting the supernatant (1x10^6 cells/ml) by centrifuging the cells at 3500 rpm for 5 min. For the control medium S2 cells were cultured as described above. WCM or CM was added to Kc cells for 24 hr

to induce Wnt/Wg signaling.

RNA-sequencing

All pair-end sequencing was performed on an Illumina HiSeq2500 machine at the Genomics Platform of the University of Geneva. For all experiments we compared three independent biological replicates and merged them for the subsequent analysis.

Computational analysis

All deep-sequencing data were mapped to the *Drosophila* reference genome dm3 using TopHat and analyzed as described in Trapnell using the Cufflinks workflow (Trapnell et al., 2012). We used R for all statistical analysis and combined with Adobe Illustrator for plotting.

Chapter 4

Conclusion

Our data demonstrate that in *Drosophila* cells all Wnt/Wg transcriptional outputs absolutely dependent on both, Arm and Pan, confirming the veracity of the Wnt/Wg signaling dogma. Pan is absolutely required for the induction of Wnt/Wg-responsive enhancers, arguing against the existence of an Arm- or Pan-independent Wnt/Wg signaling output in the tested system. As highly active enhancers were prominently associated with traditional TCF/Pan binding sites in combination with short Helper sites, similar to the combination of traditional Helper sites co-occurring with short TCF/Pan motif versions, we speculate that Pan acts via Helper sites to a greater extent than so far anticipated and that a strong TCF/Pan-DNA affinity is needed for a strong enhancer activity. We also found that repressive Wnt/Wg-responsive enhancers exhibit mostly non-conventional TCF/Pan binding sites suggesting that either Pan or other factors operate via the identified sites to mediate the repressive mode of action of enhancers. Hence, further molecular studies are required to clarify the mechanism of Wnt/Wg-mediated repression. Furthermore, our data indicate that CHIR is not a reliable surrogate inductor of the Wnt/Wg response, as CHIR stimulation could not restate the full Wg-triggered transcriptional output. As we found among the few common target genes several well-studied Wnt/Wg target genes, we anticipate that CHIR is useful for target gene confirmation rather than genome-wide approaches.

Appendix A

Appendix

A.1 Protocol for cell cloning using feeder cells

1. Healthy cultures of 5 x 10^7 *Drosophila* Kc167 cells in 10 ml are irradiated with X-ray. It is recommended to irradiate the same cell line as the cells to be cloned.
2. Transfer the cells into a 50 ml collection tube
3. 26 Kr (260 Gy) was found to be a safe dose for Kc lines. However, it is highly recommended to test prior single cell cloning various ionizing radiation doses (e.g. 360 Gy, 300 Gy and 260 Gy) to find the optimal dose to convey feeder cells in the undifferentiated state. Irradiated cells can be tested in 6 well plate and observed for 5-10 days for potential colony forming.
4. Count irradiated cells to 50.000 cells/0.1ml and pour cells into 96 well.
5. Dilute cells to be cloned to 1 cell in 0.05 ml.
6. Add cell to be cloned onto feeder cells into the 96 wells.
7. To minimize evaporation, seal the lids of the plates with e.g. parafilm.
8. Periodically inspect plates for colony formation.
9. Tiny whitish spots are easily identifiable on the bottom of the wells, among the sparse fragments of the dead feeder layer.
10. As soon as the first colonies are detectable (after around 2 weeks) let clone grow until it occupies a large portion of the well.

11. Add a drop of fresh medium after 2 weeks.

12. Each clonal line is collected by pipetting and gradually transferred into culture wells of increasing sizes.

A.2 Protocol for cell cloning using Terazaki plates

1. For single cell cloning use throughout all steps a mixture of 50% fresh and 50% conditioned medium, supplemented with 10% FBS and 1% P/S (hereafter called mixed medium). Prepare the medium always fresh at the same day when it is needed.

2. Conditioned medium is collected from *Drosophila* Kc167 cells grown over at least 2 days with a starting density of 10^6 cells/ml by centrifuging cells at 3000 rpm for 5 min at room temperature and gently pipetting the supernatant (the conditioned medium). It is recommended to use the same cell line for collecting the conditioned medium as the cell to be cloned derived from.

3. Cells to be cloned are diluted to 3 cells per 25 μl mixed medium, as on average one cell out of three survives. It is highly recommended to test how many cells should be seeded into the Terazaki plate to achieve optimal cloning efficiencies. This might vary among cell lines.

4. Pipet gently the drop of 3 cells in 25 μl mixed medium into the Terazaki well.

5. After around 15 days, small cell colonies can be detected.

6. As soon as cell clones occupy the well, transfer gently clones into the next bigger well (e.g. 96 wells) by using a 1 μl-pipett and add mixed medium to the final volume of 50 μl.

7. Close the lid with e.g. parafilm to avoid desiccation.

8. Transfer gradually cell clones into culture wells of increasing size and adjust adequately the volume of the mixed medium.

Notes: Cloning efficiencies for *Drosophila* Kc167 cells are around 33%, however cells tend to die after transferring them into the next bigger plate, as they are

quiet sensitive. Therefore, this method is not highly appreciated for *Drosophila* Kc167 cell cloning.

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